An Investigation of the Tumour-Derived Glasgow Microenvironment Score and Survival in Patients with Operable Colorectal Cancer

By

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ABSTRACT

Background: Colorectal cancer poses a significant disease burden worldwide, remaining the 2nd cause of cancer related death. The TNM staging system, whilst being constantly improved, is limited by its assessment of the tumour alone and not the host. Whereas, as the knowledge base grows regarding of the consensus molecular subtypes in colorectal cancer and the different microscopic phenotypes that these may produce, further disease biomarkers are required that reflect this understanding. The Glasgow Microenvironment Score (GMS) was developed by combining assessment of two phenotypic assessments of the colorectal cancer (CRC) microenvironment, both of which have been shown to have independent prognostic significance: the immune phenotype (assessed by Klintrup-Mäkinen grade (KM)) and the mesenchymal phenotype, assessed by tumour stroma percentage (TSP). However, further understanding of the pathological mechanisms underlying these phenotypic features is required.

Methods: The present thesis examines the prognostic utility of the GMS in several Scottish cohorts in order to validate the score in independent patient cohorts and also to assess its utility in detecting disease recurrence and understand its relevance in the context of current chemotherapy.

Results: In chapter 3, associations between markers of Epithelial-mesenchymal Transition (EMT) and the Glasgow Microenvironment Score were assessed. GMS 0 was associated with lower membrane Fascin and also lower membrane and nuclear B-catenin. GMS 1 was associated with high cytoplasmic Fascin, whereas GMS 2 was associated with higher nuclear B-catenin, a hallmark of EMT.

In Chapter 4, several cohorts were examined in order to validate the GMS in independent cohorts, including the patients from the Scot chemotherapy trial (TransScot cohort). The GMS was found to stratify survival in all of these cohorts. Furthermore, GMS 2 was found to be a risk factor for poor survival in an otherwise low-risk group.

In Chapter 5, the role of GMS in predicting disease recurrence patterns was assessed both for CRC as a whole and also in colon and rectal cancer individually. GMS independently predicted recurrence at any location for CRC and also for rectal cancers, although in colon cancers alone, this was not independent. GMS was also able to predict local recurrence, but not independently of T-stage and N-stage. GMS 2 had the highest risk for recurrence and therefore enhanced surveillance in this subgroup is recommended.

Associations between GMS and chemotherapy was assessed in Chapter 6. Standard chemotherapy did not appear to be particularly effective against GMS 2 tumours, although this data had its limitations and requires to be assessed in other cohorts. In the TransScot cohort, survival of patients with GMS 0 was better with FOLFOX compared with CAPOX.

Finally, in Chapter 7, the role of PDL1 (CD274) in prognosis of colorectal cancer and also in terms of response to immunotherapy in current trials. Whilst the expression of CD274 on immune cells was associated with good prognosis, expression on tumour tissue was equivocal in terms of survival outcomes. There is insufficient evidence regarding CD274 as a marker of response to immunotherapy in CRC and this needs to be addressed moving forward.

Conclusions: GMS has been validated in independent patient cohorts and shown to stratify survival as in the original cohort. GMS 2 has utility both in identifying patients at high-risk of disease recurrence, but also potentially in selecting patients for specific chemotherapy regimen.

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DECLARATION

The work that is presented in this thesis was carried out during a period of research between 2018 and 2020 in the Academic Unit of Colorectal Surgery, Glasgow Royal Infirmary through the MVLS college at Glasgow Royal Infirmary. I, hereby, declare that all the work contained was undertaken by me, except for the following:

- The scoring KM and TSP in the combined JP-AP TMA dataset and also the TransSCOT dataset was performed by Antonia Roseweir.
- Data analysis for the TransScot cohort was also performed primarily by Antonia Roseweir.
- The staining and scoring of MMR markers in the AP array was performed by Arfon Powell, whereas that in the JP array was performed by James Park.
- The EMT marker staining for the combined JP-AP TMA was performed by Niphat Jirapongwattana, supervised by Jean Quinn.
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- Calculating novel data thresholds for markers of EMT on R studio was performed by Kathryn Pennel.
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- Maintaining the GRI dataset was a joint effort of Clinical Research Fellows past and present and my thanks goes to my predecessors and fellow research fellows.

Ethical approval for the work contained in this thesis was provided by:

- West of Scotland Research Ethics Committee: GN170N474; 18/WS/0001; 16/WS0207 and 07/S0703/136.

PUBLICATIONS

- Alexander PG, McMillan DC, Park JH. **The local inflammatory response in colorectal cancer - Type, location or density? A systematic review and meta-analysis.** Cancer Treat Rev, 2020 Feb; 83:101949. Doi: 10.1016/j.ctrv.2019.101949
- Alexander PG, McMillan DC, Park JH. **A meta-analysis of CD274 (PD-L1) assessment and prognosis in colorectal cancer and its role in predicting response to anti-PD-1 therapy.** Crit Rev Oncol Hematol, 2021 Jan; 157:103147. doi: 10.1016/j.critrevonc.2020.103147
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- Alexander PG, van Wyk H, Pennel KAF, Hay J, McMillan DC, Horgan PG, Roxburgh CSD, Edwards J, Park, JH. **The Glasgow Microenvironment Score and risk and site of recurrence in TNM I–III colorectal cancer.** Br J Cancer, 2022 Dec; doi: 10.1038/s41416-022-02069-x

PRESENTATIONS

- **Validation of the Glasgow Microenvironment Score (GMS) in patients with colon cancer.** Oral presentation at the West of Scotland Surgical Association (November, 2019)
- **Local inflammatory response in colon cancer – indicative of good or poor prognosis?** Poster presented at ASCO GI, San Francisco (January, 2020)
- **Validation of the Glasgow Microenvironment Score in patients with colon cancer: A pathology-based prognostic tool.** Poster presented at ASCO GI, San Francisco (January, 2020)
- **The Glasgow Microenvironment Score predicts recurrence location in colorectal cancer.** Oral presentation at ACPGBI (virtual), Harrogate (July, 2021)

DEFINITIONS/ABBREVIATIONS

5-FU – 5-fluorouracil

A2aR – adenosine A2a receptor

ALDH1 – aldehyde dehydrogenase 1

APC – Adenomatous Polyposis Coli

ASA – American Society of Anesthesiologists physical status classification

AUC – area under the ROC curve

BMI – body mass index

BRAF – v-raf murine sarcoma viral oncogene homolog B1

BSA – bovine serum albumin

Ca19-9 – cancer antigen 19-9

CAPOX – chemotherapy regimen of Capecitabine and Oxaliplatin

CD (as in CD3 or CD274) – cluster of differentiation cell surface protein complex

CEA – carcinoembryonic antigen

CI – Confidence interval

CIMP – CpG Island Methylator Pathway

CIN – Chromosomal instability

CLR – Crohn's-like reaction

CMS – consensus molecular subtypes

cMyc – proto-oncogene located on chromosome 8

Cox-2 – cyclooxygenase-2 enzyme

CPS – combined positive score

CRC – Colorectal cancer

CRIS – colorectal cancer intrinsic subtypes

CRP – C-reactive Protein

CSS – Cancer-specific survival

CTLA-4 – Cytotoxic T lymphocyte-associated antigen (immune checkpoint protein)

CXCR4/7 - C-X-C chemokine receptor type 4/type 7

DAB – diaminobenzidine

DFS – Disease-free survival

Dmax – maximum tumour diameter

DPX – Dibutylphthalate Polystyrene Xylene

DSS – Disease-specific survival

ECOG – Eastern Cooperative Oncology Group score for performance status

EGFR – Epidermal growth factor receptor

EMT – epithelial-mesenchymal transition

EMVI – extramural venous invasion

ERK – extracellular signal-regulated kinase

FAP – Familial Adenomatous Polyposis syndrome

FFPE – formalin-fixed paraffin-embedded pathology specimen

FH – family history

FOLFOX – chemotherapy regimen of Folinic acid, 5-FU and Oxaliplatin

FoxP3 – Forkhead box protein P3

G-A – Graham-Appelman score of CLR

GI – gastrointestinal tract

GMS – Glasgow Microenvironment Score

GPOL – Glasgow Precision Oncology Laboratory

GRI – Glasgow Royal Infirmary

GrzB – Granzyme B

H&E – haematoxylin and eosin staining

HER2 – human epidermal growth factor receptor 2

HLA – human leukocyte antigen system

HMGB1 – High mobility group box 1 protein

HNPCC – Hereditary non-polyposis colorectal cancer

HR – Hazard ratio

I^2 – statistical test for heterogeneity of data

ICC – intraclass correlation coefficient

IDEA – International Duration Evaluation of Adjuvant Therapy trial

IDO1 – indoleamine 2,3-dioxygenase 1

IE – Intraepithelial compartment

IHC - immunohistochemistry

IM – Invasive Margin

imCMS – image-based Consensus Molecular Subtypes

IQR – interquartile range

IRORR – immune-related objective response rate

IT – Intratumoural compartment

Ki67 – nuclear marker indicating cell proliferation

KM – Klintrup-Mäkinen grade

KRAS – Kirsten rat sarcoma virus gene

K-Ras – protein encoded by KRAS

LAG3 – lymphocyte-activation gene 3

LI – lymphatic invasion

LINE1 – Long interspersed nuclear element-1

LMR – lymphocyte-monocyte ratio

LN – lymph node

LVI – lymphovascular invasion

M1/M2 – phenotype of macrophage subtypes

MAPK – mitogen-activated protein kinase

MCC – mutated in colorectal cancer

MDT – multidisciplinary team

mGPS – modified Glasgow Prognostic Score

MLH1 – MutL protein homolog 1

MMR – Mismatch repair (MMR protein coding genes).

MPE – molecular pathology epidemiology

MPR – major pathological response

MSH2 – MutS Homolog 2 (MMR protein coding genes)

MSI – microsatellite instability

MSS – microsatellite stable

MV – multivariate analysis

N or *n* – Number

NI – neural invasion

NK – natural killer cell

NLR – neutrophil-lymphocyte ratio

NPS – neutrophil-platelet score

NSAIDs – non-steroidal anti-inflammatory drugs

ORR – Objective Response Rate

OS – Overall survival

P or *p* – *p*-value

p27 – tumour suppressor protein

p53 – tumour suppressor protein

pCR – pathological Complete Regression

PD1 (also PDCD1) – Programmed cell death protein-1

PDL1 – Programmed death ligand 1

PH – proportional hazard

PI3K – Phosphatidylinositol 3 kinase protein encoded by PIK3CA

PIK3CA – Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha gene

PLR – platelet-lymphocyte ratio

PMS1/2 – PMS homolog 1/2 (MMR protein coding genes)

R1 – incomplete resection of tumour

RFA – radiofrequency ablation

RFS – Relapse-free survival

RHAMM – Receptor for hyaluronan-mediated motility

RO (as in CD45RO) – RO isoform of antigen

ROC – receiver operating characteristic curve

SCOT – Short Course Oncology Therapy study

SE – Standard error of the mean

SemiQ – semiquantitative

SOX2 – SRY-Box Transcription Factor 2

SSL – sessile serrated lesion

ST – stromal compartment

TGFB – transforming growth factor beta

TILs – tumour infiltrating lymphocytes

TMA – tissue microarray

TNM – Tumour, Node, Metastasis staging system

TP53 – Gene encoding tumour protein p53

TransSCOT – translational arm of the SCOT trial

Treg – regulatory T-cell

TRG – tumour regression grade

TSP – Tumour stromal percentage

TPS – tumour percentage score

uPA – Urokinase plasminogen activator

UV – univariate analysis

VEGF – vascular endothelial growth factor

VELIPI – venous/lymphatic/perineural invasion

VI – venous invasion

WNT – Wingless/Integrated signal transduction pathway

X^2 – Pearson Chi-squared

XELOX – another name for CAPOX

Zeb-1 – Zinc finger-E-box binding homeobox 1

DEDICATION

To my wife, Ruthie, and our three wonderful children: Noah, Caleb and Bonnie. Thanks for your patience and support as I struggled through the writing process for my thesis. Thanks for always being there to lift my spirits!

To my mum, for showing me what hard work looks life, even though I have struggled to emulate that over the years. “You’re an inspiration, mate”.

My last and greatest dedication is to the God who made me and the Lord Jesus Christ who redeemed me and set me apart for him. Without Him, none of this would be possible.

SUMMARY

Colorectal cancer represents a significant risk in terms of both morbidity and mortality on a worldwide basis. In spite of improvements in both surgical technique and available chemotherapeutic regimens, a significant proportion of patients die within 5 years of diagnosis. Current management of colorectal cancer patients relies on the TNM staging system. This system is constantly being updated, currently in its 8th rendition. However, evidence would suggest that it cannot fully account for mortality even with the addition of other clinicopathological high-risk features. The knowledge of different consensus molecular subtypes is growing in colorectal cancer and these different subtypes have been recognised to bear distinct phenotypes. The Glasgow Microenvironment Score (GMS) was developed by combining two phenotypic assessments of the colorectal cancer (CRC) microenvironment and may be a useful biomarker in order to bridge the gap between molecular subtypes and simple histopathological assessment. GMS combines an assessment of the immune phenotype, by Klintrup-Mäkinen grade (KM), and the mesenchymal phenotype, by tumour stroma percentage (TSP).

A meta-analysis of the role of different inflammatory assessments in colorectal cancer confirmed that H&E-based assessments can prognosticate survival at least as well as immunohistochemical assessments of individual inflammatory cells.

The GMS was originally performed in a relatively small cohort of 307 stage I-III colorectal cancers and was found to stratify survival into 3 distinct survival bands. However, further validation is required in independent patient cohorts.

Furthermore, the link between EMT and TSP is far from clear, as is the role of markers of EMT in the immune phenotype. In chapter 3, therefore, the role of markers of EMT was assessed with particular regard to associations with GMS phenotypes. This found evidence of novel associations of EMT markers with each GMS category, including a lower

membrane and nuclear B-catenin in GMS 0, whilst membrane B-catenin was highest in GMS 2. GMS 1 also had evidence of high cytoplasmic Fascin, the relevance of which is unclear.

In Chapter 4, the GMS was validated in a number of independent cohorts. Furthermore, associations between GMS and subgroups according to nodal status and MMR were performed to further assess the utility of GMS in each subgroup. GMS was able to stratify survival in all of the cohorts assessed. GMS 2 was found to be a high-risk phenotype in disease that would otherwise be classed as low-risk and should be considered for inclusion among other high-risk features used in clinical practice.

Chapter 5 details the assessment of GMS in relation to risk of disease recurrence. GMS 0 was found to be low-risk for recurrence (but not no risk). GMS 2 had the highest recurrence risk and therefore should be considered as a feature that may require more enhanced surveillance to detect recurrences early. GMS was an independent risk factor for developing recurrence at any location. However, for local disease recurrence, GMS was not independent of T-stage or N-stage.

The relationship between GMS and chemotherapy was assessed in Chapter 6. In the first cohort, this analysis was limited by lack of data regarding chemotherapy type and duration. However, of those patients who received adjuvant chemotherapy with GMS 2, there did not appear to be any significant survival advantage over those who did not. This adds weight to the hypothesis that GMS 2 tumours do not respond well to standard chemotherapy regimens. A more comprehensive assessment was subsequently performed in the TransScot cohort comparing GMS stages with chemotherapy type and duration. GMS 0 was the only category in which chemotherapy type made a difference. Those patients with GMS 0, who received FOLFOX appeared to have a survival benefit over those who received CAPOX. There was no association between GMS and chemotherapy duration in this cohort.

Finally, in Chapter 7, the prognostic utility of PDL1 (CD274) in colorectal cancer and also the predictive ability of CD274 for response to immunotherapy were assessed in a meta-analysis of studies and trails to date. CD274 was associated with better outcomes when assessed on inflammatory cells, but the results were equivocal in terms of survival when assessed on tumour tissue. There is currently insufficient evidence regarding the use of CD274 in the assessment of disease response to immunotherapies in colorectal cancer trials to date.

1. INTRODUCTION

1.1 COLORECTAL CANCER EPIDEMIOLOGY

Colorectal cancer (CRC) was the 3rd commonest cancer worldwide in 2020 with 1.9 million cases and was the 2nd leading cause of cancer-related mortality, responsible for 916 000 deaths the same year(World_Health_Organisation). CRC becomes more prevalent with advancing age, with 89% of new colorectal cancer cases in the USA in 2017 diagnosed in people 50 years old and above. Furthermore, 64% of CRC-related mortality in this same population was accounted for in those 65 years and over(Siegel et al., 2017).

However, age is not the only risk factor for developing colorectal cancer. The are many other factors influencing the development of cancer and these can broadly be divided into environmental and genetic factors. Several environmental factors are known to increase carcinogenesis in general, including smoking, obesity, diet, and more recently the microbiome. These factors are believed to combine in a manner that causes oxidative stress in the gastrointestinal tract, leading to mutations in the DNA sequences responsible for regulating the cell cycle, thereby leading to disordered cell replication and eventually the development of cancer(Hamada et al., 2019, Ogino et al., 2018).

1.2 MANAGING PATIENTS WITH COLORECTAL CANCER

A diagnosis of colorectal cancer is achieved with tissue sampled from the tumour and histologically verified in the histopathology laboratory(Glynne-Jones et al., 2017). This tissue is usually obtained at colonoscopy prior to further treatment, but in some cases, if presenting as an emergency, the diagnosis is made by surgical resection of the cancer and subsequent verification in the laboratory(Teixeira et al., 2015).

Colorectal cancers that present as emergencies tend to have worse survival outcomes, generally as a result of more advanced stage disease(Teixeira et al., 2015), since those with earlier stage disease will have a greater chance at cure and therefore have better 5-year survival rates compared with later stage disease (5-year survival is 90% for stage I, vs 80%

for stage II, 70% for stage III, 40% for stage IV with a solitary resected liver metastasis(Morris et al., 2010) and 10% for widespread disease)(Cancer_Research_UK, 2020). The bowel-screening programme in the United Kingdom has seen a significant improvement in detection of CRC at an earlier stage with fewer patients presenting as an emergency(Mansouri et al., 2015).

The mainstay of management of colorectal cancer remains primary surgical resection, with consideration of other treatment modalities as adjuncts to surgery. The exceptions to this are those patients who are not able to have resection due to high disease burden with widespread (and therefore palliative) disease, those patients with rectal tumours who either have disease which threatens the circumferential resection margin and require neo-adjuvant chemoradiotherapy in the first instance, and those who, having undergone neo-adjuvant treatment for rectal cancer with complete pathological response (around 20%), are then able to enter a protocolled Watch and Wait regimen(De Rosa et al., 2015, Glynne-Jones et al., 2017). In addition, there are many trials ongoing studying the use of immunotherapy as a neo-adjuvant treatment in conjunction with other treatments and modalities both in the context of colon and rectal cancer(Alexander et al., 2021).

In the UK, treatment of colorectal cancer is agreed in a multi-disciplinary team meeting (MDT) comprising surgeons, oncologists, radiologists, pathologists and colorectal specialist nurses. Cases are discussed with the results of both radiological and pathological investigations and a plan agreed after discussion and consensus. The main deciding factor in the MDT is the TNM staging system, currently in its 8th edition(Loughrey et al.), with additional clinicopathological features such as venous invasion, tumour perforation, involved resection margins (R1 resection) or low lymph node sample (<10-12). The clinicopathological features just described as adjuncts to the TNM system were combined into a score by the Gloucester Colorectal Cancer study(Petersen et al., 2002) and, although not referred to by name in most MDTs, the Petersen Index's component parts remain

important as high-risk features in decisions regarding the need for adjuvant chemotherapy. Furthermore, genetic factors, such as MSI, KRAS, and BRAF are becoming increasingly important in management planning, since they indicate which chemotherapeutic regimens the tumour is likely to respond to.

There have been many advances in the underlying pathophysiology of the disease process in recent years and these changes have led to the 8th rendition of the TNM(Loughrey et al.). Yet there remain significant discrepancies in outcomes of patients within the same TNM stage. A recent large cross-sectional study assessing the relative impact of TNM on survival among 3 cohorts of clinical trial patients found that there was considerable variability in survival that was unexplained by TNM alone. Furthermore, this was only modestly improved following the addition of other clinicopathological features such as venous invasion and mutational status(Dienstmann et al., 2017).

1.3 GENETIC MUTATIONS IN COLORECTAL CANCER

In addition to environmental factors, there are now many genetic mutations that are known to play a role in the development of colorectal cancer. These may be sub-divided into hereditary or sporadic mutations. The former include familial adenomatous polyposis (FAP, linked with mutations in chromosome 5q21 MCC and APC (Adenomatous Polyposis Coli) genes(Nishisho et al., 1991)), lynch syndrome (formerly hereditary non-polyposis colon cancer (HNPCC), but known to affect extra-colonic organs also), in addition to other inherited colorectal polyp syndromes such as Peutz-Jeghers syndrome and familial juvenile polyposis syndrome. Lynch syndrome is characterised by inherited mutations in the DNA mismatch repair genes MSH2, MLH1, PMS1 and PMS2(Lynch et al., 1997). Since the DNA mismatch repair genes are mutated, they are unable to detect the insertion of repeating DNA sequences (microsatellites) in several genes, hence the term Microsatellite Instability used to describe these tumours(Lynch et al., 1997).

Sporadic mutations responsible for colorectal cancer can occur in the same genes that are responsible for hereditary colorectal cancer. In addition to those already mentioned, other common genes and genetic pathways commonly altered in colorectal cancer include the following cell cycle genes: K-RAS is an oncogene and mutation typically results in a reduced ability for the KRAS protein to switch between active and inactive states resulting in an increased rate of mitosis(Liu et al., 2011), whereas the TP53 gene is a tumour suppressor gene responsible for arresting the cell cycle if abnormalities are identified and inducing cell senescence or apoptosis. Mutations in this gene therefore lead to failure to detect and terminate defective tumour cells(Mantovani et al., 2019). Activation of the ERK MAPK cascade by oncogenes, for example upregulation of EGFR expression, or mutational activation of BRAF and KRAS genes, is responsible for a significant proportion of spontaneous colorectal cancer(Roberts and Der, 2007). Mutations in the BRAF gene (V600E) are also known to cause MSI, but in sporadic cancers, rather than hereditary(Boland and Goel, 2010).

Generally speaking, there are three broad genetic pathways referred to in colorectal carcinogenesis: the Chromosomal Instability pathway (CIN); the Microsatellite Instability pathway (MSI) and the Serrated pathway (also known as the CpG Island Methylation Pathway, CIMP pathway)(Mundade et al., 2014).

1.3.1 Chromosomal Instability Pathway

The chromosomal instability pathway is so called because it results from abnormal numbers of chromosomes, known as aneuploidy. This pathway is reported to make up around 80% of colorectal cancers. First proposed by Fearon and Vogelstein in 1990(Fearon and Vogelstein, 1990), a stepwise pattern of mutations results in a gathering number of mutations in the cell cycle. By the sequential acquisition of chromosomal abnormalities, Fearon and Vogelstein described the progression from normal mucosa to adenomata and subsequently, over time, to carcinomata. The process begins with loss of tumour suppressor gene APC. Following

this, mutational activation of proto-oncogenes, such as KRAS and c-Myc, leads to adenoma formation and thereafter, further mutational inactivation of tumour suppressor genes, for example TP53 and the long arm of chromosome 18, result in carcinomatous transformation(Vogelstein and Kinzler, 1993). One study found that the number of major chromosomal mutations averaged around 17 per tumour(Leary et al., 2008).

1.3.2 Microsatellite Instability Pathway

Microsatellite instability (MSI) accounts for around 15% of all colorectal cancers, although around 3% are hereditary(Jenkins et al., 2007). The microsatellite instability phenotype describes the inactivation of mismatch repair genes encoding the proteins MSH2, MLH1, PMS1 and PMS2(Lynch et al., 1997). When testing was first introduced for MSI, it was purely based on genetic assessment which was expensive. High density of tumour infiltrating lymphocytes was one of the markers used in the screening process to select patients for testing(Jenkins et al., 2007), since MSI cancers were known to be immunogenic(Phillips et al., 2004, Maby et al., 2015, Dolcetti et al., 1999) – a feature of MSI tumours that has subsequently been attributed to high neo-antigen load(Wagner et al., 2018). With the advent of immunohistochemical assessment for loss of DNA-mismatch repair proteins, it has become more economical to assess for the presence of MSI in all colorectal tumours to guide adjuvant therapy(Kawakami et al., 2015).

Colorectal cancers arising from the MSI pathway have been found to be predominantly located within the proximal colon, characterised by a high lymphocytic infiltrate, less likely to metastasize and generally have a better prognosis than MSI low or microsatellite stable (MSS) colorectal cancers(Kloor et al., 2014).

1.3.3 Serrated Pathway

The serrated pathway, so called due to the colonoscopic appearance of these cancers and their precursors, is a relatively recently acknowledged phenomenon. However, serrated

carcinomas were first described in 1992 by Jass and Smith(Jass and Smith, 1992). These make up 15-30% of colorectal cancers with overlapping features of both the CIN pathway and the MSI pathway(Satorres et al., 2021). The molecular mechanisms underlying the serrated pathway include the CpG Island Methylation Pathway (CIMP), BRAF, MSI and the mitogen-activated protein Kinase (MAPK) pathway(Satorres et al., 2021, Tornillo et al., 2021).

The precursors to these lesions are hyperplastic polyps, which have been found to have a high rate of BRAF mutation. These may progress, through a series of methylations, mostly due to CpG Island Methylation Pathway (CIMP) to: Sessile Serrated Lesions (SSLs), with or without dysplasia, if BRAF is the predominant mutation; or tubular serrated adenomata, if KRAS is the predominant mutation(Satorres et al., 2021).

Whilst the presence of BRAF and CIMP are hallmarks of SSLs, the presence of MSI is a late development and once MSI and dysplasia are present, progression to a serrated pathway colorectal cancer is believed to take place relatively rapidly(Satorres et al., 2021).

1.4 CONSENSUS MOLECULAR SUBTYPES

In some cancers, such as breast, it has been possible to categorise different tumours according to mutational status (for example by the presence or absence of the oestrogen receptor, progesterone receptor or Her-2 receptor), which also aids the selection of adjuvant therapy required(Cortés et al., 2011). In CRC, however, there are not such clear-cut mutational distinctions, but rather a plethora of genetic mutations and drivers involved in carcinogenesis with significant overlap between the agreed pathways of cancer development. An attempt was made to group colorectal cancers into categories according to mutational status in 2015, known as the Consensus Molecular Subtypes (CMS)(Guinney et al., 2015b). These were designated group numbers 1-4: CMS 1 represents an inflammatory cell-rich microenvironment and encompasses “hypermutated” tumours, including those with MSI,

CIMP etc; CMS 2 represents the canonical subtype, with chromosomal instability and activation of WNT and MYC signalling pathways; CMS 3 was named the metabolic subtype with evidence of "metabolic dysregulation"; and CMS 4 represents the mesenchymal subtype, with tumours in this category rich in stroma and high tumour budding(Guinney et al., 2015b).

However, attempting to fit patients into a specific CMS based purely on mutational data is not possible as the original CMS study found. Some of the individuals with MSI, CIMP, BRAF and known to be hypermutated were designated CMS 2-4 and there were many with TP53, KRAS and APC mutations that were designated CMS 1. Furthermore, there were 10-15% of colorectal cancers that could not be assigned to a specific CMS subtype. However, MSI, CIMP and BRAF were significantly higher in CMS 1(Guinney et al., 2015b).

In a study of the associations between KRAS and CMS, Lal et al. found that there was suppression of inflammatory pathways in CMS 2, CMS 3 and KRAS mutant tumours (Lal et al., 2018). Becht et al. also found that immune and inflammatory signatures, while being prominent in CMS 1, were suppressed in CMS 2 and 3(Becht et al., 2016).

Arguably, all of these assessments stage the tumour itself, rather than the interaction between tumour and host. This has been identified as a shortfall of published literature in the past and several phenotypic assessments of the tumour microenvironment have been developed to combat this(Park et al., 2016b, Park et al., 2016a).

There are also phenotypic differences that might be utilised to define the different CMS categories. CMS 1 has been denoted the Immune subtype and will therefore have higher immune scores than the other subtypes. Whereas, CMS 4 has been denoted the Mesenchymal subtype and therefore histological features of mesenchymal tumours, such as tumour stroma or tumour budding could identify this subtype(Becht et al., 2016). Furthermore, CMS classifications have been shown to predict response to chemotherapy(Testa et al., 2020).

The two colorectal cancer phenotypes that lend themselves most readily to assessment are the immune phenotype, corresponding with CMS 1 and the mesenchymal phenotype, corresponding to CMS 4.

However, there is no consensus currently on the optimal means of assessing the immune phenotype. This was the conclusion of the International Immuno-Oncology Biomarkers Working Group(Hendry et al., 2017) regarding the inflammatory response in CRC and it is likely to be a significant factor in the lack of progress in clinical practice in this field in the last decade. In the latest edition of the CRC reporting dataset in the UK (TNM 8), assessment of the local inflammatory response remains only an optional item(Loughrey et al.). There are some who are calling for the addition of an immune category into the TNM system(Pagès et al., 2018, Galon et al., 2014).

Therefore, a systematic review and meta-analysis was performed with the aim of identifying the optimal method of scoring the inflammatory infiltrate in the colorectal cancer tumour microenvironment(Alexander et al., 2020b).

1.5 OPTIMUM INFLAMMATORY CELL SCORING METHODOLOGY

One area of discord among experts in the field is whether assessment using standard H&E-stained slides is sufficient to assess the response or whether immunohistochemistry may lend a superior prognostic capability and, if so, which inflammatory cell markers should be assessed. A further contentious area is whether the specific tumour compartment in which there is a higher inflammatory cell density has a greater prognostic role: at the invasive margin, within the tumour stroma or within the cancer cell nests. There are those who advocate that assessment of intratumoural lymphocytes may give superior prognostic data(Galon and Bruni, 2019). Others still have suggested the tumour may, in some instances, employ immune escape mechanisms in order to evade the host's immune system. This may take the form of a physical barrier that has been described as a "basement membrane-like"

structure(Menon et al., 2004), or it may manifest in the expression on tumour cells the Programmed Cell Death Ligand 1 (CD274)(Rosenbaum et al., 2016).

Therefore, it was important to establish whether or not an assessment of intratumoural inflammatory cells was required in order to accurately assess prognosis or whether an assessment of inflammatory cells at the invasive margin of the tumour would suffice.

Furthermore, one of the confounding factors in many studies is the assessment or lack, thereof, of the presence or absence of MSI. MSI tumours are known to be immunogenic(Maby et al., 2015) due to a high neo-antigen load(Wagner et al., 2018). Therefore, the presence of MSI, with its associated immunogenicity may confound prognostic results since MSI itself is considered largely indicative of good prognosis.

1.5.1 Specific Methods

1.5.1.1 Search Strategy

The following search terms were entered into PubMed, Ovid, MEDLINE and EMBASE databases:

- "colorectal cancer" or "colon cancer" or "rectal cancer" (Abstract) AND
- "prognos$" or "survival" AND
- "immunohistochemistry" (any field) AND

• "KM" or "Klintrup-Mäkinen" or "CLR" or "Crohn's-like reaction" or "peritumo$ inflamm$" (Abstract)	**OR**	• "Cytotoxic" or "CD8" or "CD3" or "CD4" or "T-cell" or "Tcell" or "lymphocyte" or "macrophage" or "CD68" or "CD163" or "natural killer" or "CD56" or "CD57" or "CD45RO" or "FoxP3" or "Treg" or "T-reg" or "CD20" or "tumo$ infiltrating lymphocytes" or "TILS" (Abstract)

The search was limited to English language articles, published after 1997 and human studies. Search was performed by PGA and all primary studies were identified via abstracts +- review of the main article for data extraction. A search was also made of the reference sections of the articles identified to assess if any studies had been overlooked in the literature search. Inclusion criteria: prospective or retrospective design, but a well-defined study population; primary cancer resections for colon, rectal or colorectal cancer; use of FFPE slides and either standard H&E-staining or IHC staining for specific inflammatory cell markers; clear description in the methods of the specimen, antibodies and counting method/tumour compartment employed; description of groups assessed and thresholds employed; statistical analysis method; and, in the case of meta-analysis, inclusion of a proportional hazards model with details of any adjustment methods. Any contentious articles were discussed with DCM or JHP to agree those for inclusion.

1.5.1.2 Data Extraction

Generic data on the year of study and clinicopathological data on the individual patient population(s) were extracted. Further important data extracted included type of specimen studied, whether tissue microarray (TMA, and size of core, if given) or full resection specimen, method of inflammatory infiltrate assessment, which immunohistochemical stain was used (if any), MSI status of tumours and treatment of this in the statistical analysis, which survival outcome was used (cancer-specific, overall, disease-free, etc.) and Hazard ratios/p-values if provided. All relevant studies were included in literature review, but only studies that performed multivariate analysis with hazard ratios were included in meta-analysis. Figure 1.1 shows the flow diagram of studies used in systematic review and meta-analysis.

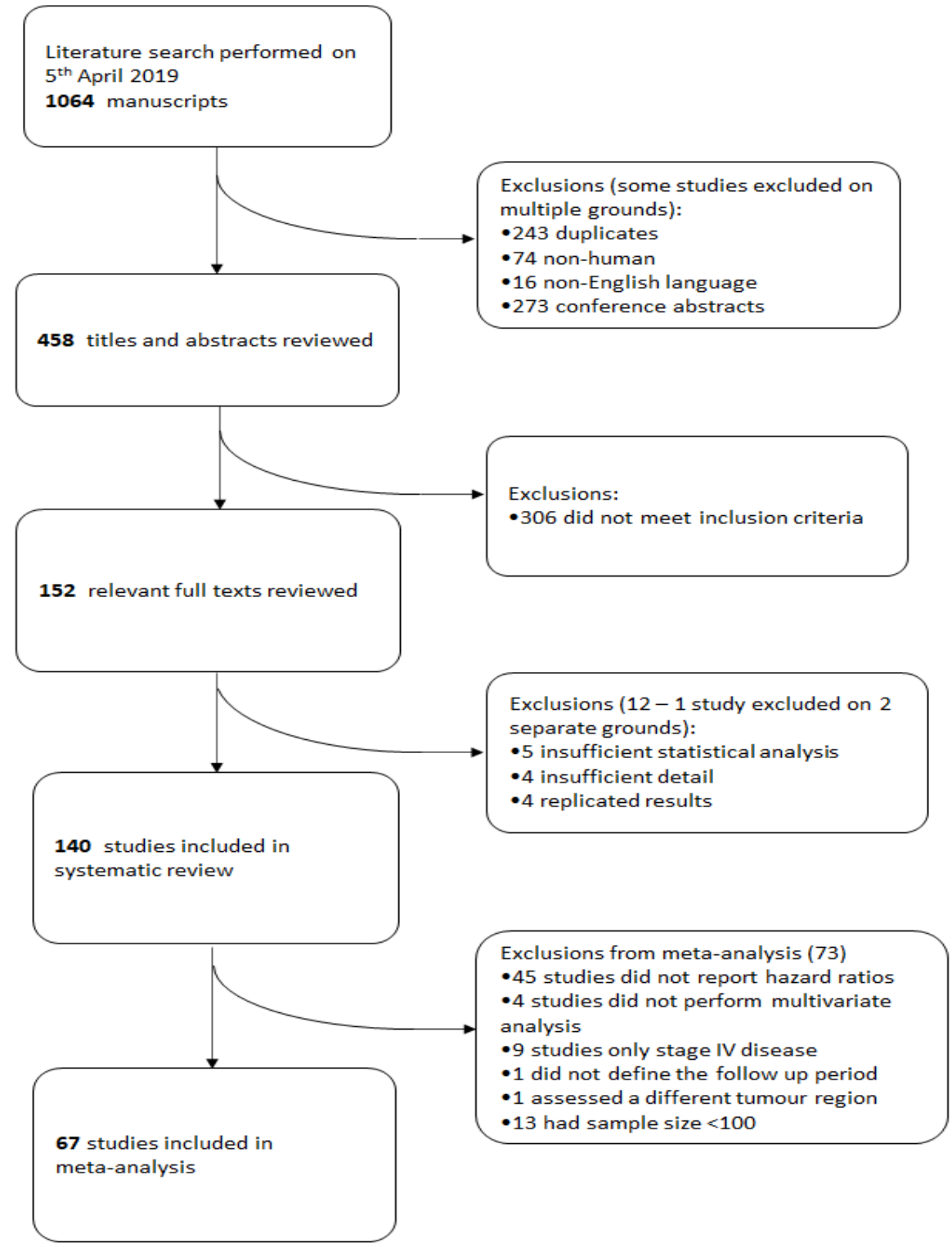


Figure 1.1. Flow diagram indicating reasons for excluding studies from systematic review and meta-analysis

1.5.1.3 Statistical analysis

For all inflammatory assessments, studies were grouped according to tumour location i.e., colon, rectal or colorectal. Further, studies were collated according to the specific method of survival analysis: disease-free survival (DFS) was defined as the length of time between diagnosis and recurrence or spread of disease ("recurrence-free survival" and "progression-free survival" were also grouped under this heading); overall survival (OS) was defined as the length of time between diagnosis and death from any cause; disease-specific survival (DSS) was defined as the length of time between diagnosis and death from CRC-related cause ("cancer-specific survival" was also included under this heading). Furthermore, the individual location of inflammatory cells assessed was treated in the following way: since one of the main questions was whether "intratumoural" (IT) inflammatory cell assessment was superior to assessment at the "invasive margin" (IM), studies assessing inflammatory cell populations in either the "intra-epithelial compartment" (IE) or within the tumour stroma (ST) were group together as IT and compared against those at the IM. Of course, for those studies using an "immunoscore-type" method, assessing both IM and IT compartments and forming a combined score, these were included with the IT studies. Studies of small sample size ($n<100$) were excluded from meta-analysis due to potential for bias. REVMAN (version 5.3) software was used for meta-analysis. Funnel plots were used to assess the potential for publication bias, while forest plots and I^2 values were used to assess inter-study heterogeneity. Multiple studies in the same category are presented with a fixed effects summary HR and 95% CI, whereas in the event of only a single study being included in a particular category, the HR and 95% CI are given for that study. Confidence intervals were considered not significant where they crossed 1.0.

1.5.2 Overview of Studies

1.5.2.1 Search results and exclusions

The initial literature search (see Figure 1.1) yielded 1064 titles, of which 243 were duplicates, 74 were non-human, 16 non-English language and 273 were conference abstracts only. Abstracts were viewed for the remaining 458 studies after exclusions. A further 306 were found not to meet inclusion criteria and there remained 152 studies for which full texts were scrutinized. Of these 12 were excluded for insufficient detail, insufficient statistical analysis or replication of results, leaving 140 studies for the systematic literature review (see Table 1.1 and Appendix 1 for tables representing these studies).

Table 1.1. Summary table of studies included in literature review reporting survival outcomes based on peritumoural inflammatory infiltrate and inflammatory cell subtypes

Measurement of local inflammatory response	Total number of studies[a]	Studies reporting significant positive association[a] (%)	Studies reporting significant negative association[a] (%)	Studies reporting no survival association[a]
Inflammatory infiltrate on H&E				
Klintrup-Mäkinen/Jass	18	14 (78)		4 (22)
CLR	14	13 (93)		1 (7)
TILs (H&E)	9	8 (89)		1 (11)
Combined assessment	2	2 (100)		0
Any H&E method	**32**	**30 (94)**		**2 (6)**
T-lymphocyte subsets				
CD3 (generic T-cell)	34	24 (71)		10 (29)
CD8 (cytotoxic T-cell)	62	46 (74)		16 (26)
CD4 (helper T-cell)	15	6 (40)		9 (60)
CD45RO (memory T-cell)	15	12 (80)		3 (20)
FoxP3 (regulatory T-cell)	34	21[b] (62)	2[b] (6)	12 (35)
Combined T-cells	**86**	**67[b] (78)**	**2[b] (2)**	**18 (21)**
Immunoscore	14	13 (93)		1 (7)
B-lymphocytes (CD20)	6	5 (83)		1 (17)
Natural killer cells (CD56, CD57)	6	5 (93)		1 (7)
Macrophages (CD68, CD163, CD206)				
CD68	17	10 (59)	1 (6)	6 (45)
CD163	7	2 (29)	3 (43)	2 (28)
CD206	1	0	1 (100)	0
Combined macrophages	**22**	**12 (55)**	**5 (23)**	**5** (22)
Total	**140**	**119[c] (85)**	**7[c] (5)**	**16** (11)

[a]numbers in columns will not add up as many studies looked at more than one marker
[b]one study both positive and negative
[c]two studies reported both positive and negative findings

For the meta-analysis, a further 73 studies were excluded for the following reasons: lack of hazard ratios (n=45); no multivariate analysis (n=4); only stage IV disease (n=9); no defined follow up period (n=1); alternative tumour region (n=1); or small sample size, <100 (n=13). This left 67 studies, a summary of which is presented in Table 1.2. A bias assessment was also performed and is shown in Table 1.3. Only one included study had a moderate risk of bias and the remaining studies were of low-risk.

1.5.2.2 Study characteristics

In terms of the specimens used in those studies included in the meta-analysis, 35 used whole resection specimens, 29 used TMAs and 3 used a combination of TMAs, whole sections and biopsies. TMA core sizes ranged from 0.6mm to 3mm. Twenty-one studies employed automated cell counts, whilst 46 used a manual method of cell counting. Blinding was only explicitly stated for 27 studies, whereas sample sizes ranged from 103 up to 2681 patients/tumours. Most studies (n=51) had less than 500 tumours and the median study size was 285 (IQR 160 – 478). The presence of MSI was assessed in 69% of studies, although only 74% of those assessing MSI included it in multivariate analysis. More than 45 adjustment variables were employed in multivariate analyses. The most common of these was age (n=45), with gender and tumour grade being the next commonest (n= 37 and 35 respectively). Other commonly used variables included N-stage, TNM, T-stage, MSI, tumour site, lympho-vascular/perineural invasion and other inflammatory assessments.

From the outset, it is important to note that the presence of MSI was handled poorly in many studies, but that even where MSI was included in survival analysis, it was often found to have no prognostic value. Furthermore, the inflammatory response often had prognostic value that was independent of the presence of MSI.

Table 1.2. Summary of studies included in meta-analysis, cell markers assessed and methodology

First author, year (ref)	Colon (C) Rectal (R) Colorectal (CR)	Section assessed	Counting site	Threshold	Marker assessed	Anti-body	Blind-ing	Adjustment variables	Study size	Surv-ival
Alderdice 2017(Alderdice et al., 2017)	R	TMA 1mm	IT (combined)	Arbitrary	CD56	Novocastra	NA	Age, sex, TNM, EMVI, TRG	149	**OS**
Algars 2012(Algars et al., 2012)	CR	TMA 1.2mm	IT (ST)	Arbitrary	CD68	Abcam	Y	Stage, sex, Inflammatory cell markers	159	**DSS**
Bae 2011(Bae et al., 2011)	CR	TMA 2mm and Whole	IM & IT (IE)	Unclear	CLR CD8	 Dako	NA	Age, stage, grade, CLR/KM/CD8TILs, Chemo	169	**OS**
Baker 2007(Baker et al., 2007)	CR	TMA 0.6mm	IT (IE)	ROC	CD8	Dako	NA	EMT markers (3), TGFB (2), Ki67, MSI	150	**DSS**
Berntsson 2016(Berntsson et al., 2016)	CR	TMA 1mm	IT (combined)	ROC	CD20	Ventana	NA	Age, sex, T-stage, N-stage, M-stage, grade, VI	542	**OS**
Berntsson 2017(Berntsson et al., 2017)	CR	TMA 1mm	IT (combined)	ROC	CD3 CD8 FoxP3	Ventana Dako Abcam	NA	Age, sex, T-stage, N-stage, M-stage, grade, VI	541	**OS**
Buckowitz 2005(Buckowitz et al., 2005)	CR	Whole	IM	SemiQ	CLR		Y	Age, stage, MSI, adjuvant chemo	118	**DFS** **OS**
Chen 2016(Chen et al., 2016)	CR	TMA 1mm	IT (combined)	ROC	CD3 CD8 CD4 CD45RO FoxP3 CD20 CD57 CD68	ZSGB-BIO ZSGB-BIO ZSGB-BIO ZSGB-BIO ZSGB-BIO ZSGB-BIO ZSGB-BIO Thermo	NA	Age, sex, tumour size, stage, inflammatory cells	300	**DFS** **OS**

Chen 2019b(Chen et al., 2019b)	R	TMA 2mm and Biopsy	IT (ST)	SemiQ	CD8	Abcam	Y	Age, N-stage, clinical response, TRG, PDL1	112	**DFS OS**
Chiba 2004(Chiba et al., 2004)	CR	Whole	IT (IE)	Median	CD8	Dako	NA	N-stage, M-stage, invasive pattern, age, site, sex, MSI, grade	371	**DSS**
Climent 2019(Climent et al., 2019)	CR	Whole	IM	SemiQ	KM TILs		NA	Age, sex, TNM, tumour site, NLR, LMR	173	**DFS OS**
Dahlin 2011(Dahlin et al., 2011)	CR	Whole	IM + IT	SemiQ	CD3	Dako	NA	Age, sex, tumour site, stage, adjuvant	308	**DSS**
Deschoolmeester 2010(Deschoolmeester et al., 2010)	CR	Whole	IM & IT (IE & ST)	Arbitrary	CD3 CD8	Neomarkers Novocastra	NA	Age, sex, stage, location, grade, adjuvant, other inflammatory cells/compartments	215	**DFS OS**
Edin 2012(Edin et al., 2012)	CR	Whole	IM	SemiQ	CD163	Novocastra	NA	Gender, age, tumour location	368	**DSS**
Eriksen 2018(Eriksen et al., 2018)	CR	Whole	IT (combined)	Arbitrary	CD3 CD8	Dako Dako	NA	Age, sex, T-stage, N-stage, LN count, location, grade, perf, MSI, LI, NI	573	**DFS OS**
Galon 2006(Galon et al., 2006)	CR	TMA 0.6 and 1mm	Immunoscore	ROC	CD3	Neomarkers	Y	T-stage, diff, N-stage	249	**DFS OS**
Guidoboni 2001(Guidoboni et al., 2001)	C	Whole	IT (IE)	Median	CD3 CD8	Dako Dako	NA	Age, sex, TNM, MSI	109	**DFS OS**
Gulubova 2013(Gulubova et al., 2013)	CR	Whole	IM & IT (ST)	Arbitrary	CD68	Dako	NA	Age, T-stage, N-stage, M-stage, TNM, grade, KM, VELIPI	210	**OS**
Hynes 2017(Hynes et al., 2017)	C	Whole	IM	SemiQ	KM CLR		NA	Age, sex, adjuvant therapy, TNM, year of diagnosis, family history, MSI, EMVI, ECOG, grade	445	**DSS OS**
Kasajima 2010(Kasajima et al., 2010)	CR	TMA 1.5mm	IT (combined)	SemiQ	CD8 CD4	Dako Novocastra	NA	Age, stage, N-stage, M-stage, grade, another inflammatory cell	285	**OS**

Kim 2015a(Kim et al., 2015a)	CR	Whole	IM	Arbitrary	CLR		Y	Age, stage, grade, CIMP	212	**DFS**
Kim 2015b(Kim et al., 2015b)	CR	TMA 2mm	IM & IT (combined)	Median	CD8 CD45RO FoxP3	Neomarkers Neomarkers Abcam	NA	TNM, LI, VI, 3 inflammatory cell markers	767	**DFS** **OS**
Kim 2018(Kim et al., 2018)	CR	TMA 2mm	IT (IE & ST)	Median	CD3 CD8 CD68 CD163	Dako Neomarkers Dako Leica	NA	TNM, LI, VI, NI, inflammatory cells/locations (3)	654	**DFS** **OS**
Klintrup 2005(Klintrup et al., 2005)	CR	Whole	IM	SemiQ	KM		Y	Stage, tumour location, sex	228	**DFS** **OS**
Koelzer 2016(Koelzer et al., 2016)	CR	TMA 0.6mm	IT (IE & ST)	Mean	CD68 CD163	Dako Novocastra	NA	T-stage, N-stage, M-stage, adjuvant	201	**OS**
Laghi 2009(Laghi et al., 2009)	CR	Whole	IM	ROC	CD3	Dako	Y	Age, sex, MSI, tumour site, T-stage, N-stage, grade, VI, adjuvant	119	**DFS** **DSS**
Lee 2016(Lee et al., 2016)	CR	Whole	IM & IT (IE & ST)	Arbitrary	CLR TILs		NA	Age, sex, grade, medullary, mucinous, site, T-stage, N-stage, TNM, TILs/KM, PD1/PDL1	391	**DFS**
Li 2018a(Li et al., 2018a)	CR	TMA 1.5mm	IT (combined)	Mean	CD68	Dako	NA	Age, sex, T-stage, N-stage, Grade, site, Diabetic, HTN	216	**OS**
Ling 2014(Ling et al., 2014)	CR	Whole	IM + IT	Arbitrary	CD8 FoxP3	Cell Signal. Tc. Abcam	NA	Age, sex, stage, site	257	**DSS**
Lugli 2009(Lugli et al., 2009)	CR	TMA 0.6mm and Whole	IM	ROC	CD8	Dako	NA	Age, sex, T-stage, N-stage, grade, VI, tumour border	455	**DSS**
Matsutani 2018(Matsutani et al., 2018)	CR	Whole	IM & IT (IE & ST)	Median	CD8 CD4	Dako Dako	Y	Age, T-stage, Histology type, LI, VI, N-stage, CEA, CA19-9, MSI	313	**DSS**
Miller 2017(Miller et al., 2017)	C	TMA 1mm	IM & IT (IE & ST)	ROC	CD3 CD8 FoxP3	Ventana Dako Abcam	Y	T-stage, N-stage, grade, mucinous, ALDH1, SOX2,	104	**OS** **DSS**

								another inflammatory cell marker (CD3/FoxP3)		
Mlecnik 2011(Mlecnik et al., 2011)	CR	TMA 0.6 and 1mm	Immunoscore	ROC	IS (CD45RO + CD8)	Neomarkers Neomarkers	Y	Sex, T-stage, N-stage, LN count, grade, mucinous, perforation, obstruction	341	**DFS OS DSS**
Mori 2015(Mori et al., 2015)	CR	Whole	IT (IE & ST)	Median	CD8 FoxP3	GeneTex Abcam	NA	Age, sex, T-stage, N-stage, grade, LI, VI, CEA, CRP, NLR, PLR, another inflammatory cell marker	157	**DFS**
Naito 1998(Naito et al., 1998)	CR	Whole	IM & IT (IE & ST)	SemiQ	CD8	Dako	NA	Stage, KM, invasion pattern, histology type	129	**OS**
Nazemalhosseini-Majorad 2019(Nazemalhosseini-Mojarad et al., 2019)	CR	Whole	IT (IE & ST)	Arbitrary	CD8	Dako	NA	Age, stage, MSI, FH, CD8 location	281	**OS**
Nielsen 1999(Nielsen et al., 1999)	CR	Whole	IT (combined)	Arbitrary	TILs		Y	Age, stage, location, other inflammatory cells x2	588	**OS**
Nosho 2010(Nosho et al., 2010)	CR	TMA 0.6mm	IM + IT	Arbitrary	CD3 CD8 CD45RO FoxP3	Dako Dako Dako BioLegend	NA	Age, sex, BMI, FH, year diagnosed, tumour location, grade, CIMP, MSI, BRAF, KRAS, PIK3CA, LINE1, 3 other immune markers	727	**OS DSS**
Ogino 2009(Ogino et al., 2009)	CR	Whole	IM & IT (IE)	SemiQ	KM CLR TILs		Y	Age, year of diagnosis, sex, FH, tumour location, tumour grade, KRAS, BRAF, MSI, LINE1, p53, CIMP	846	**DSS OS**
Oshikiri 2006(Oshikiri et al., 2006)	CR	Whole	IT (combined)	Unclear (likely median)	CD8	Dako	Y	Age, sex, grade, LI, VI, stage	146	**OS**
Pagès 2009(Pagès et al., 2009)	CR	TMA 0.6 and 1mm	Immunoscore	ROC	CD8 CD45RO	Neomarkers Neomarkers	Y	T-stage, perforation	369	**DFS OS DSS**

Pagès 2018(Pagès et al., 2018)	C	Whole	Immunoscore	Arbitrary	IS (CD3 + CD8)	Ventana Dako	Y	Age, sex, T-stage, N-stage, MSI	2681	**DFS OS**
Park 2014(Park et al., 2014)	CR	Whole	IM	SemiQ	KM		NA	Age, sex, adjuvant therapy, mGPS, tumour site, T-stage, N-stage, TSP, tumour necrosis etc	307	**DSS**
Park 2016a(Park et al., 2016a)	CR	Whole	IM + IT	SemiQ	IS (CD3 + CD8)	Vector Dako	Y	MSI, mGPS, NPS, other inflammatory cell (2),	246	**DSS**
Prall 2004(Prall et al., 2004)	CR	TMA (unclear size)	IT (IE & ST)	Arbitrary	CD8	Dako	NA	Stage, adjuvant therapy, MSI	152	**DFS DSS**
Prizment 2017(Prizment et al., 2017)	CR	TMA (unclear size)	IM & IT (combined)	Arbitrary	CD8	Dako	NA	Age, stage, BMI, smoking, grade	565	**OS DSS**
Reimers 2014(Reimers et al., 2014)	R	TMA 1mm	IT (combined)	Median	FoxP3	Abcam	Y	TNM, stage, CRM, age, grade, adjuvant therapy	478	**DFS OS**
Richards 2014(Richards et al., 2014)	CR	Whole	IM & IT (IE & ST)	SemiQ	CD3 CD8 CD45RO FoxP3	Vector Labs Dako Dako Abcam	Y	Other lymphocyte subsets, KM and immunoscore	329	**DSS**
Ropponen 1997(Ropponen et al., 1997)	CR	Whole	IM + IT (ST)	Arbitrary	TILs		NA	Stage, Dmax, Nuclear area, grade	195	**DFS DSS**
Rosenbaum 2016(Rosenbaum et al., 2016)	R	TMA 2mm	IT (IE)	SemiQ	CD8	Leica	Y	Age, sex, T-stage, N-stage, PDL1, MSI, BRAF, KRAS, medullary	180	**DSS**
Rozek 2016(Rozek et al., 2016)	CR	Whole	IM & IT (IE)	Arbitrary	CLR TILs		Y	Age, sex, ethnicity, MSI, stage, grade	2268	**OS DSS**
Salama 2009(Salama et al., 2009)	CR	TMA 1mm	IT (IE)	Median	CD8 CD45RO FoxP3	Dako Dako Abcam	Y	Stage, tumour site, grade, VI, LI, PI, TILs, MSI, other immune cell markers (5)	967	**OS**

Shibutani 2017a(Shibutani et al., 2017a)	CR	Whole	IM	Median	CD163	Leica	Y	Age, sex, T-stage, grade, LI, VI, N-stage, CEA, CA19-9	168	**DFS OS**
Simpson 2010(Simpson et al., 2010)	CR	TMA 0.6mm	IT (IE & ST)	Mean	CD3	Neomarkers	Y	Stage, EMVI	555	**DSS**
Sinicrope 2009(Sinicrope et al., 2009)	C	TMA (unclear size)	IT (IE & ST)	Arbitrary (percentile)	CD3 FoxP3	Dako Abcam	NA	Age, N-stage, grade, MSI, adjuvant	160	**DFS OS**
Tachibana 2005(Tachibana et al., 2005)	CR	Whole	IT (IE)	ROC	CD56 CD57	Dako Dako	Y	Age, sex, site, grade, LI, VI, T-stage, N-stage, M-stage	103	**DFS OS**
Tosolini 2011(Tosolini et al., 2011b)	CR	TMA 0.6mm	IT (combined)	ROC	CD8	Neomarkers	NA	Age, T-stage, N-stage, site, grade, mucin, obstruction, perforation	103	**DFS**
Turner 2016(Turner et al., 2016)	C	Whole	IT (combined)	SemiQ	TILs		Y	NLR, T-stage, LVI, age, ASA, MSI	396	**DFS OS**
Ueno 2013(Ueno et al., 2013)	CR	Whole	IM	Arbitrary	CLR		NA	T-stage, N-stage, grade, VI, budding	1354	**DSS**
Ueno 2015(Ueno et al., 2015)	CR	Whole	IM	Arbitrary	CLR		Y	Sex, T-stage, N-stage, tumour size, Lymph Node count, budding, desmoplastic reaction, Site, VI, Adjuvant therapy	1354	**DFS**
Väyrynen 2012(Väyrynen et al., 2012)	CR	Whole	IM + IT	ROC	CD3	Leica	Y	Age, sex, grade, T-stage, N-stage, tumour site, budding	235	**DSS**
Väyrynen 2014(Väyrynen et al., 2014)	CR	Whole	IM	SemiQ/ROC for CLR	KM CLR		NA	TNM, grade, tumour location, KM or CLR, MMR	329	**DSS**
Väyrynen 2016(Väyrynen et al., 2016)	CR	TMA 3mm	IM & IT (IE & ST)	ROC	CD3 CD8 FoxP3 CD68	Novocastra Novocastra Abcam Dako	Y	Age, T-stage, N-stage, tumour location, neoadjuvant, M-stage, MSI, other inflammatory cell markers (2-3)	147	**DFS OS DSS**

Wirta 2017(Wirta et al., 2017)	CR	TMA 0.6mm	Immunoscore	ROC	IS (CD3+ CD8)	Novocastra Thermo	NA	Age, sex, stage, PI, LI, MSI, BRAF, tumour site	417	**DFS OS DSS**
Yoon 2012(Yoon et al., 2012)	C	TMA (unclear size)	IT (IE & ST)	Median	CD8 FoxP3	Dako Abcam and Dako	NA	Age, stage, grade, MMR	156	**OS**
Zlobec 2008a(Zlobec et al., 2008a)	R	TMA 0.6mm	IT (combined)	ROC	CD8	Dako	NA	T-stage, N-stage, age, RHAMM	458	**DSS**
Zlobec 2008b(Zlobec et al., 2008b)	CR	TMA 0.6mm	IT	ROC	CD8	Dako	NA	p27, uPA, tumour classification	587	**OS**

Table 1.3. Assessment of bias for studies included in meta-analysis[a].

First author, year (ref)	Population well defined (selection bias)	Method of inflammatory assessment specified (selection bias)	Threshold defined (selection bias)	Group allocation defined (observer bias)	Blinding (observer bias)	Evaluation by >1 observer (observer bias)	Loss to follow up (attrition bias)	Patient and tumour characteristics (reporting bias)	Follow up defined/ Specified (reporting bias)	Risk of bias[a]	Bias High/ med/low
Alderdice 2017(Alderdice et al., 2017)	1	**1**	**1**	**1**	**0**	**1**	**1**	**1**	1	**8**	**Low**
Algars 2012(Algars et al., 2012)	0 (no dates)	**1**	**1**	**0**	**1**	**1**	**1**	**1**	1	**7**	**Low**
Bae 2011(Bae et al., 2011)	1	**1**	**1**	**1**	**0**	**1**	**1**	**1**	1	**8**	**Low**
Baker 2007(Baker et al., 2007)	1	**1**	**1**	**1**	**0**	**0**	**1**	**1**	0	**6**	**Low**
Berntsson 2016(Berntsson et al., 2016)	1	**1**	**1**	**1**	**0**	**0**	**1**	**1**	1	**7**	**Low**
Berntsson 2017(Berntsson et al., 2017)	1	**1**	**1**	**1**	**0**	**0**	**1**	**1**	1	**7**	**Low**
Buckowitz 2005(Buckowitz et al., 2005)	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	1	**9**	**Low**
Chen 2016(Chen et al., 2016)	1	**1**	**1**	**0**	**0**	**1**	**1**	**1**	1	**7**	**Low**
Chen 2019(Chen et al., 2019b)	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	0	**8**	**Low**

Chiba 2004(Chiba et al., 2004)	0 (no dates)	**1**	**1**	**1**	**0**	**1**	**1**	**1**	1	**7**	**Low**
Climent 2019(Climent et al., 2019)	1	**1**	**1**	**1**	**0**	**0**	**1**	**1**	1	**8**	**Low**
Dahlin 2011(Dahlin et al., 2011)	1	**1**	**1**	**1**	**0**	**0**	**1**	**1**	1	**7**	**Low**
Deschoolmeester 2010(Deschoolmeester et al., 2010)	1	**1**	**1**	**1**	**0**	**1**	**1**	**1**	1	**8**	**Low**
Edin 2012(Edin et al., 2012)	1	**1**	**1**	**1**	**0**	**0**	**1**	**1**	0	**7**	**Low**
Eriksen 2018(Eriksen et al., 2018)	1	**1**	**1**	**1**	**0**	**1**	**1**	**1**	1	**8**	**Low**
Galon 2006(Galon et al., 2006)	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	1	**9**	**Low**
Guidoboni 2001(Guidoboni et al., 2001)	1	**1**	**1**	**0**	**0**	**0**	**1**	**1**	1	**6**	**Low**
Gulubova 2013(Gulubova et al., 2013)	1	**1**	**1**	**1**	**0**	**0**	**1**	**1**	1	**7**	**Low**
Hynes 2017(Hynes et al., 2017)	1	**1**	**1**	**1**	**0**	**1**	**1**	**1**	1	**8**	**Low**
Kasajima 2010(Kasajima et al., 2010)	1	**1**	**1**	**1**	**0**	**0**	**1**	**1**	1	**7**	**Low**
Kim 2015a(Kim et al., 2015a)	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	1	**9**	**Low**
Kim 2015b(Kim et al., 2015b)	1	**1**	**1**	**0**	**0**	**1**	**1**	**1**	0	**6**	**Low**

Kim 2018(Kim et al., 2018)	1	**1**	**1**	**1**	**0**	**1**	**0**	**1**	0	**6**	**Low**
Klintrup 2005(Klintrup et al., 2005)	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	1	**9**	**Low**
Koelzer 2016(Koelzer et al., 2016)	1	**1**	**1**	**1**	**0**	**1**	**0**	**1**	0	**6**	**Low**
Laghi 2009(Laghi et al., 2009)	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	1	**9**	**Low**
Lee 2016(Lee et al., 2016)	0 (no dates)	**1**	**1**	**1**	**0**	**0**	**0**	**1**	1	**6**	**Low**
Li 2018a(Li et al., 2018a)	0 (no dates)	**1**	**0**	**1**	**0**	**1**	**1**	**1**	1	**6**	**Low**
Ling 2014(Ling et al., 2014)	1	**1**	**1**	**1**	**0**	**1**	**1**	**1**	1	**9**	**Low**
Lugli 2009(Lugli et al., 2009)	1	**1**	**1**	**1**	**0**	**0**	**1**	**1**	1	**7**	**Low**
Matsutani 2018(Matsutani et al., 2018)	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	1	**9**	**Low**
Miller 2017(Miller et al., 2017)	1	**1**	**1**	**1**	**1**	**0**	**1**	**1**	1	**8**	**Low**
Mlecnik 2011(Mlecnik et al., 2011)	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	0	**8**	**Low**
Mori 2015(Mori et al., 2015)	1	**1**	**1**	**1**	**0**	**1**	**0**	**1**	1	**7**	**Low**
Naito 1998(Naito et al., 1998)	1	**1**	**1**	**1**	**0**	**0**	**0**	**0**	0	**4**	**Mod**
Nazemalhosseini-	1	**1**	**1**	**1**	**0**	**1**	**1**	**1**	1	**8**	**Low**

Majorad 2019(Nazemalhosseini-Mojarad et al., 2019)											
Nielsen 1999(Nielsen et al., 1999)	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	1	**9**	**Low**
Nosho 2010(Nosho et al., 2010)	1	**1**	**1**	**1**	**0**	**1**	**1**	**1**	1	**8**	**Low**
Ogino 2009(Ogino et al., 2009)	1	**1**	**1**	**1**	**1**	**1**	**0**	**1**	0	**7**	**Low**
Oshikiri 2006(Oshikiri et al., 2006)	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	1	**9**	**Low**
Pagès 2009(Pagès et al., 2009)	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	1	**9**	**Low**
Pagès 2018(Pagès et al., 2018)	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	1	**9**	**Low**
Park 2014(Park et al., 2014)	1	**1**	**1**	**1**	**0**	**1**	**1**	**1**	1	**8**	**Low**
Park 2016a(Park et al., 2016a)	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	1	**9**	**Low**
Prall 2004(Prall et al., 2004)	1	**1**	1	1	0	0	1	1	1	**7**	**Low**
Prizment 2017(Prizment et al., 2017)	1	**1**	**1**	**1**	**0**	**1**	**1**	**1**	1	**8**	**Low**
Reimers 2014(Reimers et al., 2014)	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	0	**8**	**Low**
Richards 2014(Richards et al., 2014)	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	1	**9**	**Low**
Ropponen 1997(Ropponen et al., 1997)	1	**1**	**1**	**1**	**0**	**1**	**1**	**1**	1	**8**	**Low**

Rosenbaum 2016(Rosenbaum et al., 2016)	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	0	**8**	**Low**
Rozek 2016(Rozek et al., 2016)	1	**1**	**1**	**1**	**1**	**0**	**1**	**1**	0	**7**	**Low**
Salama 2009(Salama et al., 2009)	1	**1**	**1**	**0**	**1**	**1**	**1**	**1**	1	**8**	**Low**
Shibutani 2017a(Shibutani et al., 2017a)	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	0	**8**	**Low**
Simpson 2010(Simpson et al., 2010)	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	1	**9**	**Low**
Sinicrope 2009(Sinicrope et al., 2009)	1	**1**	**1**	**1**	**0**	**1**	**1**	**1**	0	**7**	**Low**
Tachibana 2005(Tachibana et al., 2005)	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	1	**9**	**Low**
Tosolini 2011(Tosolini et al., 2011b)	1	**1**	**1**	**1**	**0**	**1**	**1**	**1**	0	**7**	**Low**
Turner 2016(Turner et al., 2016)	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	1	**9**	**Low**
Ueno 2013(Ueno et al., 2013)	1	**1**	**1**	**1**	**0**	**0**	**1**	**1**	1	**7**	**Low**
Ueno 2015(Ueno et al., 2015)	1	**1**	**1**	**1**	**1**	**0**	**1**	**1**	1	**8**	**Low**
Väyrynen 2012(Väyrynen et al., 2012)	1	**1**	**1**	**0**	**1**	**1**	**1**	**1**	0	**7**	**Low**

Väyrynen 2014(Väyrynen et al., 2014)	1	**1**	**1**	**0**	**0**	**1**	**1**	**1**	0	**6**	**Low**
Väyrynen 2016(Väyrynen et al., 2016)	1	**1**	**1**	**0**	**1**	**1**	**1**	**1**	1	**8**	**Low**
Wirta 2017(Wirta et al., 2017)	1	**1**	**1**	**1**	**0**	**1**	**1**	**1**	1	**8**	**Low**
Yoon 2012(Yoon et al., 2012)	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	1	**9**	**Low**
Zlobec 2008a(Zlobec et al., 2008a)	1	**1**	**1**	**1**	**0**	**0**	**1**	**1**	1	**7**	**Low**
Zlobec 2008b(Zlobec et al., 2008b)	1	**1**	**1**	**0**	**0**	**1**	**1**	**1**	0	**6**	**Low**

[a]Assessment of bias table score developed from REMARK guidelines[23], total out of 9: scores of 0-3 were considered high-risk for bias; scores of 4 or 5, moderate; and scores of 6 and above, low-risk.

1.5.3 H&E-Based Scoring of Local Inflammatory Response

There were 32 studies identified that employed an H&E-based assessment of peritumoural inflammation in colorectal cancer. These methods included that described by Jass, Klintrup-Mäkinen grade (KM), Crohn's-like reaction (CLR), tumour infiltrating lymphocytes (TILs) or a method combining these. Of these, 4 study cohorts overlapped(Bae et al., 2011, Klintrup et al., 2005, Richards et al., 2012, Ueno et al., 2015), leaving 28 independent studies and a total of 11,423 patients(Menon et al., 2004, Szynglarewicz et al., 2007, Hynes et al., 2017, Turner et al., 2016, Harrison et al., 1995, Coca et al., 1997, Nagtegaal et al., 2001, Huh et al., 2012, Shibutani et al., 2018, Climent et al., 2019, Cianchi et al., 2002, Gao et al., 2005, Ogino et al., 2009, Kasajima et al., 2010, Xie et al., 2018, Chiba et al., 2004, Park et al., 2014, Väyrynen et al., 2014, Ropponen et al., 1997, Lee et al., 2016, Iseki et al., 2018, Nielsen et al., 1999, Rozek et al., 2016, Lang-Schwarz et al., 2018, Buckowitz et al., 2005, Kim et al., 2015a, Ueno et al., 2013, Wallace et al., 2018). Of these, only 2 studies did not find any significant difference for any H&E-based method(Kasajima et al., 2010, Menon et al., 2004), whilst the remaining 26 studies found that a higher local anti-tumour inflammatory response was significantly associated with better survival outcome, totalling 10,887 patients(Szynglarewicz et al., 2007, Hynes et al., 2017, Turner et al., 2016, Harrison et al., 1995, Coca et al., 1997, Nagtegaal et al., 2001, Huh et al., 2012, Shibutani et al., 2018, Climent et al., 2019, Cianchi et al., 2002, Gao et al., 2005, Ogino et al., 2009, Xie et al., 2018, Chiba et al., 2004, Park et al., 2014, Väyrynen et al., 2014, Ropponen et al., 1997, Lee et al., 2016, Iseki et al., 2018, Nielsen et al., 1999, Rozek et al., 2016, Lang-Schwarz et al., 2018, Buckowitz et al., 2005, Kim et al., 2015a, Ueno et al., 2013, Wallace et al., 2018).

1.5.3.1 Jass and Klintrup-Mäkinen grade (KM)

The Klintrup-Mäkinen grade and Jass scoring systems are related but were developed separately. Both assess the quantity of the local inflammatory infiltrate on standard Haematoxylin and Eosin (H&E)-stained slides in the context of colorectal cancer. H&E-

staining forms the basis for all standard pathological slide analysis in clinical histopathological practice. Jass et al. first reported the independent prognostic advantage in rectal cancer denoted by an increase in peritumoural inflammatory cell infiltrate in 1986(Jass, 1986). The lymphocytic infiltrate at the tumour's invasive margin (IM), or "advancing front", was described as pronounced, moderate, little or none. "Pronounced" inflammation at the IM might appear as a "cap" or otherwise continuous layer of inflammatory cells, whereas a broken or interrupted inflammatory cell layer represented "moderate" inflammation with fewer cells present overall. The categories denoted as "little" and "none" were combined into one category. This 3-point scale was found to stratify rectal cancer survival into 3 distinct bands(Jass, 1986). Klintrup, Mäkinen and colleagues(Klintrup et al., 2005) developed a similar phenotypic assessment of the local inflammatory infiltrate at the IM in 2005. Their 4-point scale scored a "cup-like" inflammatory cell infiltrate as 3, a band-like infiltrate scored 2, an interrupted band of inflammatory cells scored 1, whilst minimal inflammation scored 0. A further assessment of the destruction of cancer cell nests was required to score 2 or 3. They subsequently dichotomised the score by combining the upper two and the lower 2 categories (i.e., 0 or 1 was low, 2 or 3 was high). These H&E-based inflammatory scores have been validated by many other groups (Hynes et al., 2017, Szynglarewicz et al., 2007, Coca et al., 1997, Nagtegaal et al., 2001, Shibutani et al., 2018, Climent et al., 2019, Gao et al., 2005, Ogino et al., 2009, Xie et al., 2018, Chiba et al., 2004, Park et al., 2014, Väyrynen et al., 2014, Huh et al., 2012).

Given the similarities between the Jass and Klintrup-Mäkinen scores, the papers assessing one or the other were combined for the purposes of this systematic review and meta-analysis. There were 18 studies assessing peritumoural inflammation with this method, although one of these had an overlapping cohort(Klintrup et al., 2005), which left 17 studies assessing 4904 patients. Of these studies, four found no association with survival(Menon et al., 2004, Bae et al., 2011, Cianchi et al., 2002, Kasajima et al., 2010), whilst thirteen studies (4046

patients) found a higher peritumoural inflammatory response to be associated with longer survival(Szynglarewicz et al., 2007, Hynes et al., 2017, Coca et al., 1997, Nagtegaal et al., 2001, Huh et al., 2012, Shibutani et al., 2018, Climent et al., 2019, Gao et al., 2005, Ogino et al., 2009, Xie et al., 2018, Chiba et al., 2004, Park et al., 2014, Väyrynen et al., 2014). Interobserver variability was quoted by four separate groups. In the original study, the interobserver agreement was 0.72(Jass, 1986) and that quoted in the Klintrup-Mäkinen study was 0.50 – 0.79(Klintrup et al., 2005). Roxburgh et al.(Roxburgh et al., 2009) found interobserver agreement of 0.71 for Jass and 0.81 for KM, whereas Hynes et al(Hynes et al., 2017) found a poor interobserver agreement at best with a range of 0.05-0.48.

The presence of MSI was assessed by 9 separate studies and of these: one study only contained MSI-high tumours and found that peritumoural inflammation was not significantly associated with survival in this group(Bae et al., 2011); two studies found that peritumoural inflammation was significant for survival independent of MSI(Hynes et al., 2017, Ogino et al., 2009); three studies did not find MSI to be associated with survival(Xie et al., 2018, Chiba et al., 2004, Väyrynen et al., 2014); while three did not include MSI in survival analysis(Menon et al., 2004, Climent et al., 2019, Gao et al., 2005).

Those studies meeting inclusion criteria for meta-analysis of KM or Jass scores are given in Tables 1.4 and 1.5 (Figure 1.2). There was one study in colon cancer, which found KM to be significant for longer OS and DSS (HRs 0.63, 95% CI 0.42-0.95 and 0.48, 95% CI 0.31-0.75, respectively)(Hynes et al., 2017). There were six studies in colorectal cancer giving combined fixed effects HRs of 0.62 (95% CI 0.43-0.88, p=0.007) for disease-free survival (DFS) in 3 studies(Climent et al., 2019, Klintrup et al., 2005, Menon et al., 2004); 0.43 (95% CI 0.26-0.71, p<0.001) for overall survival (OS) in 2 studies(Climent et al., 2019, Ogino et al., 2009); and 0.40 (95% CI 0.29-0.55, p<0.001) for disease-specific survival (DSS) in 3 studies(Ogino et al., 2009, Park et al., 2014, Väyrynen et al., 2014), with no significant heterogeneity and no evidence of publication bias, although study numbers were small.

Table 1.4. Meta-analysis results for studies assessing survival (DFS, OS and DSS) in colon cancer in relation to peritumoural inflammatory assessments

Impact of study methodology on heterogeneity testing (I^2 test) and overall effect							
		Overall effect			Heterogeneity		
Location assessed	**Survival type**	**No. of studies**	**HR**	**95% CI**	**I^2 (%)**	***P*-value**	**First Author Surname/year**
Klintrup-Mäkinen/Jass							
	OS	1	0.63	0.42-0.95	NA		Hynes
	DSS	1	0.48	0.31-0.75	NA		Hynes
Crohn's-like reaction							
G-A	OS	1	0.64	0.48-0.86	NA		Hynes
	DSS	1	0.60	0.42-0.85	NA		Hynes
Tumour infiltrating lymphocytes (H&E)							
	DFS	1	0.37	0.15-0.90	NA		Turner
	OS	1	0.45	0.23-0.87	NA		Turner
CD3							
IT	DFS	2	0.59	0.38-0.91	3	0.31	**Guidoboni**, Sinicrope
	OS	3	0.49	0.33-0.71	0	0.83	**Guidoboni**, Miller, Sinicrope
	DSS	1	0.35	0.14-0.88	NA		Miller
IM	OS	1	0.48	0.22-1.03	NA		Miller
	DSS	1	0.65	0.50-0.84	NA		Miller
CD8							
IT	DFS	1	0.35	0.16-0.76	NA		**Guidoboni**
	OS	3	0.58	0.41-0.83	34	0.22	**Guidoboni**, Miller, Yoon
	DSS	1	0.77	0.32-1.87	NA		Miller
IM	OS	1	0.84	0.41-1.71	NA		Miller
	DSS	1	0.77	0.32-1.87	NA		Miller
FoxP3							
IT	DFS	1	1.23	0.72-2.13	NA		Sinicrope
	OS	2	0.91	0.59-1.40	86	0.008	Miller, Sinicrope
	DSS	1	0.28	0.12-0.66	NA		Miller
Immunoscore							
	DFS	1	0.63	0.52-0.75	NA		Pagès 18
	OS	1	0.70	0.58-0.84	NA		Pagès 18

Bold studies: Right sided tumours only

Table 1.5. Meta-analysis results for studies assessing survival (DFS, OS and DSS) in colorectal cancer in relation to peritumoural inflammatory assessments

Impact of study methodology on heterogeneity testing (I^2 test) and overall effect							
		Overall effect			Heterogeneity		
Location assessed	**Survival type**	**No. of studies**	**HR**	**95% CI**	**I^2 (%)**	***P*-value**	**First Author Surname/year**
Klintrup-Mäkinen/Jass							
	DFS	3	0.62	0.43-0.88	0	0.39	Climent, Klintrup, Menon
	OS	2	0.43	0.26-0.71	0	0.45	Climent, Ogino
	DSS	3	0.40	0.29-0.55	0	0.91	Ogino, Park 14, Väyrynen 14
Crohn's-like reaction							
G-A	DFS	1	0.87	0.26-2.89	NA		Lee 16
	OS	4	0.68	0.60-0.78	53	0.09	**Bae**, Buckowitz, Ogino, Rozek
	DSS	2	0.64	0.54-0.77	0	0.60	Ogino, Rozek
Ueno	DFS	2	0.49	0.37-0.64	0	0.98	**Kim 15a**, Ueno 15
	DSS	1	0.40	0.20-0.80	NA		Ueno 13
Väyrynen	DFS	1	0.50	0.28-0.89	NA		**Kim 15a**
	DSS	1	0.54	0.37-0.79	NA		Väyrynen 2014
Any method	DFS	3	0.51	0.39-0.66	0	0.84	**Kim 15a**, Ueno 15, Lee 16
	OS	4	0.68	0.60-0.78	53	0.09	**Bae**, Buckowitz, Ogino, Rozek
	DSS	4	0.61	0.52-0.71	0	0.50	Ogino, Rozek, Ueno 13, Väyrynen 14
Tumour infiltrating lymphocytes (H&E)							
	DFS	3	0.65	0.51-0.83	0	0.37	Climent, Lee 16, Ropponen
	OS	4	0.73	0.64-0.84	0	0.73	Climent, Nielsen, Ogino, Rozek
	DSS	3	0.66	0.55-0.78	0	0.95	Ogino, Ropponen, Rozek
Combined H&E inflammatory assessment							
	OS	1	0.50	0.31-0.81	NA		Ogino
	DSS	1	0.31	0.15-0.65	NA		Ogino
CD3							
IT	DFS	6	0.46	0.39-0.54	56	0.04	Chen 16, Deschoolmeester, Erisken, Galon 06, Kim 18, Väyrynen 16
	OS	8	0.57	0.50-0.64	73	<0.001	Berntsson 17, Chen 16, Deschoolmeester, Eriksen, Galon 06, Kim 18, Nosho, Väyrynen 16
	DSS	6	0.59	0.50-0.70	0	0.82	Dahlin, Nosho, Richards 14, Simpson, Väyrynen 12, Väyrynen 16
IM	DFS	3	0.45	0.33-0.61	0	0.69	Deschoolmeester, Galon 06, Väyrynen 16
	OS	4	0.71	0.59-0.85	67	0.03	Deschoolmeester, Galon 06, Nosho, Väyrynen 16
	DSS	6	0.58	0.48-0.69	10	0.35	Dahlin, Laghi, Nosho, Richards 14, Väyrynen 12, Väyrynen 16

CD8							
IT	DFS	8	0.46	0.39-0.54	48	0.06	Chen 16, Deschoolmeester, Eriksen, Kim 18, Mori, Prall, Tosolini, Väyrynen 16
CD8 continued							
	OS	15	0.63	0.58-0.67	65	<0.001	Berntsson 17, Chen 16, Deschoolmeester, Erisken, Kasajima, Kim 18, Naito, Nazemalhosseini-Majorad, Nosho, Oshikiri, Pagès 09, Prizment, Salama, Väyrynen 16, Zlobec 2008b
	DSS	9	0.62	0.56-0.69	73	<0.001	Baker, Chiba, Ling, Nosho, Pagès 09, Prall, Prizment, Richards 14, Väyrynen 16
IM	DFS	4	0.50	0.40-0.62	0	0.81	Deschoolmeester, Kim 15b, Tosolini, Väyrynen 16
	OS	4	0.62	0.51-0.75	58	0.05	Deschoolmeester, Kim 15b, Nosho, Väyrynen 16
	DSS	5	0.53	0.45-0.63	53	0.05	Lugli, Matsutani, Nosho, Richards 14, Väyrynen 16
CD4							
IT	DFS	1	0.55	0.32-0.96	NA		Chen 16
	OS	2	0.64	0.42-0.97	0	0.72	Chen 16, Kasajima
	DSS	1	0.64	0.41-0.99	NA		Ling
CD45RO							
IT	DFS	3	0.52	0.40-0.69	83	0.003	Chen 16, Kim 15b, Pagès 09
	OS	5	0.68	0.61-0.75	78	0.001	Chen 16, Kim 15b, Nosho, Pagès 09, Salama
	DSS	3	0.53	0.44-0.64	83	0.002	Nosho, Pagès 09, Richards 14
IM	DFS	1	0.42	0.33-0.54	NA		Kim 15b
	OS	2	0.51	0.42-0.63	77	0.01	Kim 15b, Nosho
	DSS	2	0.57	0.47-0.68	70	0.07	Nosho, Richards 14
FoxP3							
IT	DFS	2	0.52	0.36-0.77	27	0.24	Chen 16, Väyrynen 16
	OS	4	0.72	0.65-0.80	77	0.004	Chen 16, Nosho, Salama, Väyrynen 16
	DSS	3	0.47	0.37-0.61	0	0.40	Nosho, Richards 14, Väyrynen 16
IM	DFS	1	0.42	0.19-0.93	NA		Väyrynen 16
	OS	2	0.47	0.35-0.63	0	0.81	Nosho, Väyrynen 16
	DSS	3	0.57	0.46-0.70	62	0.07	Nosho, Richards 14, Väyrynen 16
Immunoscore							
	DFS	3	0.49	0.41-0.58	91	<0.001	Mlecnik, Pagès 09, Wirta
	OS	3	0.61	0.53-0.70	89	<0.001	Mlecnik, Pagès 09, Wirta
	DSS	5	0.47	0.39-0.55	86	<0.001	Mlecnik, Nearchou, Pagès 09, Park 16, Wirta
CD20							
IT	DFS	1	0.62	0.40-0.96	NA		Chen 16
	OS	2	0.66	0.52-0.89	0	0.50	Berntsson 16, Chen 16

CD57/Va24							
IT	DFS	2	0.47	0.28-0.78	71	0.06	Chen 16, Tachibana
	OS	2	0.48	0.28-0.84	33	0.22	Chen 16, Tachibana
CD68							
IT	DFS	3	1.21	0.95-1.55	81	0.005	Chen 16, Kim 18, Väyrynen 16
	OS	5	0.91	0.75-1.11	77	0.002	Chen 16, Gulubova, Kim 18, Koelzer 16, Väyrynen 16
	DSS	2	0.58	0.38-0.89	0	0.56	Algars, Väyrynen 16
IM	DFS	1	0.43	0.19-0.96	NA		Väyrynen 16
	OS	3	0.48	0.36-0.64	48	0.15	Gulubova, Li 18a, Väyrynen 16
	DSS	1	0.40	0.20-0.81	NA		Väyrynen 16
CD163/206							
IM	DFS	1	3.68	1.74-7.82	NA		Shibutani 17
	DSS	1	0.66	0.42-1.05	NA		Edin

Bold studies: MSI high only studies

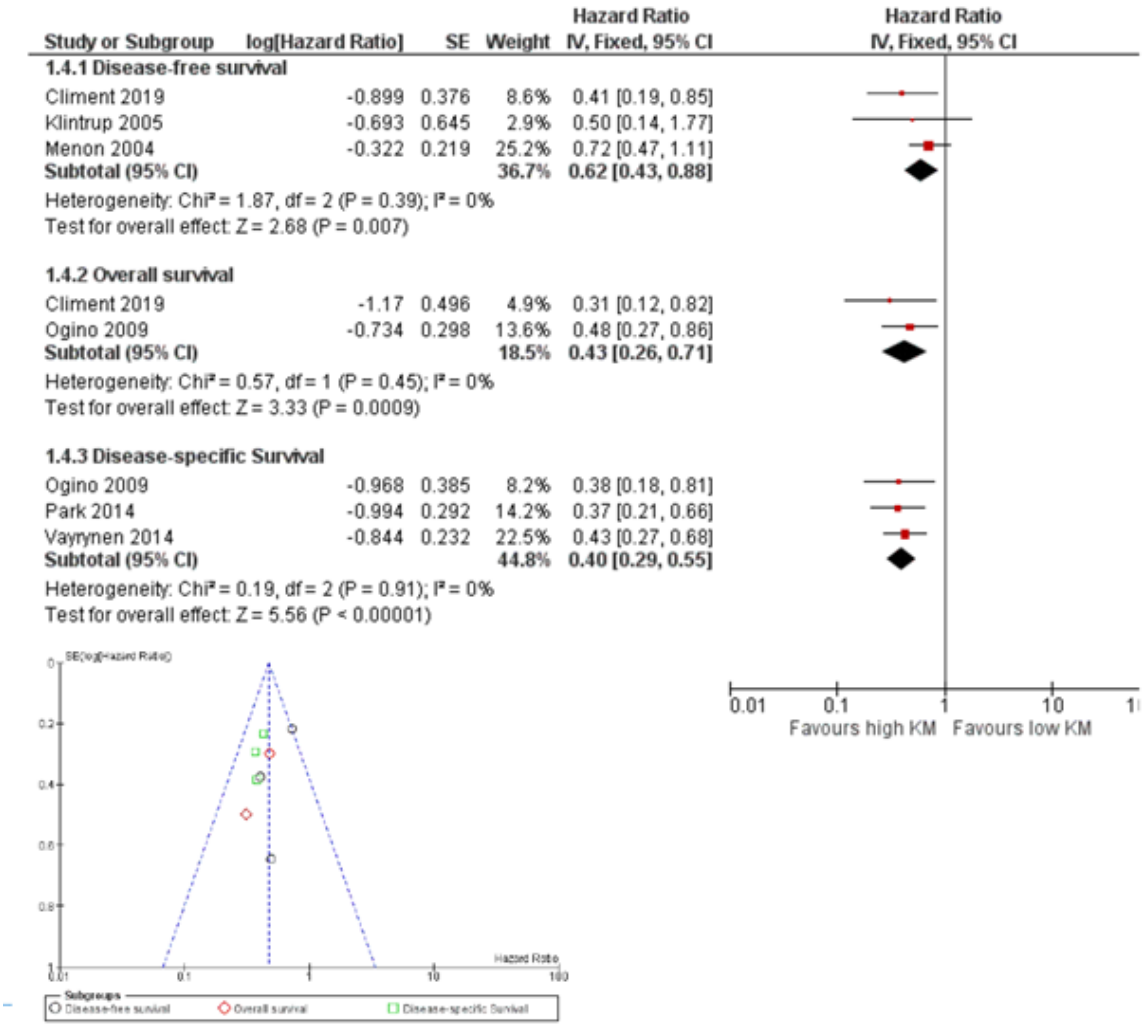


Figure 1.2. Forest plot and funnel plot for KM/Jass classification in colorectal cancer according to DFS, OS and DSS

1.5.3.2 Crohn's-Like Reaction (CLR)

Crohn's-like reaction (CLR) was originally described in 1990 by Graham and Appelman(Graham and Appelman, 1990). The score assesses how many "discrete lymphoid aggregates" are present at the IM and spread through the muscularis propria and which of these have germinal centres. The CLR is thought to represent part of the body's adaptive inflammatory response to cancer. These lymphoid aggregates have been found to consist largely of B-cells, with T-cells and antigen-presenting cells also present to a lesser degree(Väyrynen et al., 2014). According to the original method of CLR assessment, tumours than 3 or more lymphoid aggregates and at least 1 germinal centre were scored as "intense". A reaction was considered "mild" if 2 or fewer aggregates were present or "none" if there were no aggregates(Graham and Appelman, 1990). Several groups, in attempting to validate this method, have combined two of the categories, whether intense vs none or mild(Buckowitz et al., 2005, Harrison et al., 1995) or none vs mild or intense(Klintrup et al., 2005).

Two alternative methods for scoring CLR have been proposed since then. The method proposed by Ueno et al.(Ueno et al., 2013) measures the bipolar length of the largest lymphoid aggregate and tumours with aggregates longer than 1mm were considered CLR "active". The method proposed by Väyrynen et al.(Väyrynen et al., 2014) is a "density-based" assessment calculated by counting the total number of lymphoid aggregates and dividing this by the length of the invasive tumour margin measured in millimetres on the same slide used for counting aggregates. A data-driven threshold of 0.38 follicles/mm was calculated using a ROC-curve and those tumours above this threshold were considered high density. Other studies have attempted to measure B-cell response by counting plasma cells on standard H&E-stained slides either at the invasive margin(Richards et al., 2012) or within the tumour(Nielsen et al., 1999).

There were 14 studies reporting CLR or plasma cell counts, although three studies had overlapping cohorts(Bae et al., 2011, Klintrup et al., 2005, Ueno et al., 2015). This left eleven studies with 6595 patients. All of these studies found higher CLR/plasma cell counts to be significant for longer survival(Richards et al., 2012, Hynes et al., 2017, Harrison et al., 1995, Ogino et al., 2009, Väyrynen et al., 2014, Lee et al., 2016, Nielsen et al., 1999, Rozek et al., 2016, Buckowitz et al., 2005, Kim et al., 2015a, Ueno et al., 2013). In terms of individual assessment method, 7 studies with 2337 patients used the Graham-Appelman method (G-A). However, even in these studies, the threshold group for dichotomising patients was different. Four studies, of 2337 patients dichotomised by grouping the lower two groups together (i.e. absent and mild in one group and intense in the other): of these one found no significant difference in survival in a study of only MSI-high patients(Kim et al., 2015b); whereas the other 3 studies found the survival was better in the intense group(Buckowitz et al., 2005, Rozek et al., 2016, Harrison et al., 1995). Three studies, of 1466 patients, dichotomised patients with the two higher groups together (i.e. absent in one group vs mild or intense in the other): of these, one study found no significant association with survival(Klintrup et al., 2005), while the other two found a significant survival advantage(Hynes et al., 2017, Ogino et al., 2009). A further study used a modified G-A method, classing an "intense" reaction as greater than 5 lymphoid aggregates(Lee et al., 2016). However, with this classification, only one tumour met the criteria to be included in this category. Therefore, this study also effectively dichotomised with mild and intense in the same group and did not find CLR to be significant for survival.

Only one group compared different dichotomisation thresholds for CLR in the same cohort of patients, albeit in two separate papers(Kim et al., 2015a, Bae et al., 2011). The studies assessed MSI-high tumours only and where the lower threshold was used there were 144 tumour with mild or intense reactions vs 25 with an absent reaction(Bae et al., 2011), while with the higher threshold, there were 45 with an intense reaction vs 164 with absent or

mild(Kim et al., 2015a). The higher threshold was found to be significant for longer survival, while the lower threshold was not. The reason that the lower threshold was significant is likely due to the fact that this was an MSI-high cohort and the lower threshold selected out those patients with an absent local inflammatory response (in terms of CLR) in spite of what would otherwise be considered immunogenic tumour biology.

Three studies, of 2073 patients, used alternative methods of measuring CLR: one study used the Ueno method(Ueno et al., 2013); one used the Väyrynen method(Väyrynen et al., 2014); one study compared Ueno and Väyrynen methods with the G-A method(Kim et al., 2015a). All of these studies found these alternative CLR methods to be significant for longer survival.

In terms of reproducibility, the G-A method's interobserver agreement was reported as 0.29(Hynes et al., 2017), 0.50 in 2 studies(Kim et al., 2015a, Ueno et al., 2013), and 0.92(Harrison et al., 1995). The interobserver agreement reported for the Ueno method was 0.56(Kim et al., 2015a) and 0.67(Ueno et al., 2013). For the Väyrynen method, agreement ranged between 0.71 and 0.81(Väyrynen et al., 2014, Kim et al., 2015a). Only one study directly compared interobserver agreement for all three methods of scoring CLR and they found the method with the greatest agreement was the density-based Väyrynen method(Kim et al., 2015a).

Eight studies reported assessment for MSI: of which four studies found that CLR was independent of MSI(Kim et al., 2015a, Rozek et al., 2016, Ogino et al., 2009, Hynes et al., 2017); two studies found MSI was not significant for survival(Buckowitz et al., 2005, Väyrynen et al., 2014); and the other two did not assess survival according to MSI(Ueno et al., 2013, Lee et al., 2016).

Those studies meeting inclusion criteria for meta-analysis of CLR methods are given in Tables 1.4 and 1.5 (Figure 1.3). There was one study in colon cancer, which found CLR (G-A) to be significant for longer OS and DSS (HRs 0.64, 95% CI 0.48-0.86 and 0.60, 95% CI

0.42-0.85, respectively)(Hynes et al., 2017). There were five studies in colorectal cancer for CLR (G-A), with a single study finding it not significant for DFS(Lee et al., 2016); but combined fixed effects HRs of 0.68 (95% CI 0.60-0.78) for (OS) in 4 studies(Bae et al., 2011, Buckowitz et al., 2005, Ogino et al., 2009, Rozek et al., 2016); and 0.64 (95% CI 0.54-0.77) for DSS in 2 studies(Ogino et al., 2009, Rozek et al., 2016), with no significant evidence of heterogeneity for DSS. However, for OS there was evidence of moderate heterogeneity (I^2 53%, p=0.09).

There were three studies in colorectal cancer for CLR (Ueno), with a combined fixed effects HR of 0.49 (95% CI 0.37-0.64) for DFS in two studies(Kim et al., 2015a, Ueno et al., 2015) and no evidence of significant heterogeneity; but only a single study for OS finding CLR (Ueno) to be significant (HRs 0.40, 95% CI 0.20-0.80)(Ueno et al., 2013).

There were two studies in colorectal cancer for CLR (Väyrynen), with a single study assessing DFS, finding it significant (HRs 0.50, 95% CI 0.28-0.89)(Kim et al., 2015a) and a single study assessing DSS, finding it significant (HRs 0.54, 95% CI 0.37-0.79)(Väyrynen et al., 2014).

The funnel plot for CLR (G-A) did not suggest any evidence of publication bias. However, there were too few studies for the Ueno or Väyrynen methods for any reliable funnel plot analysis.

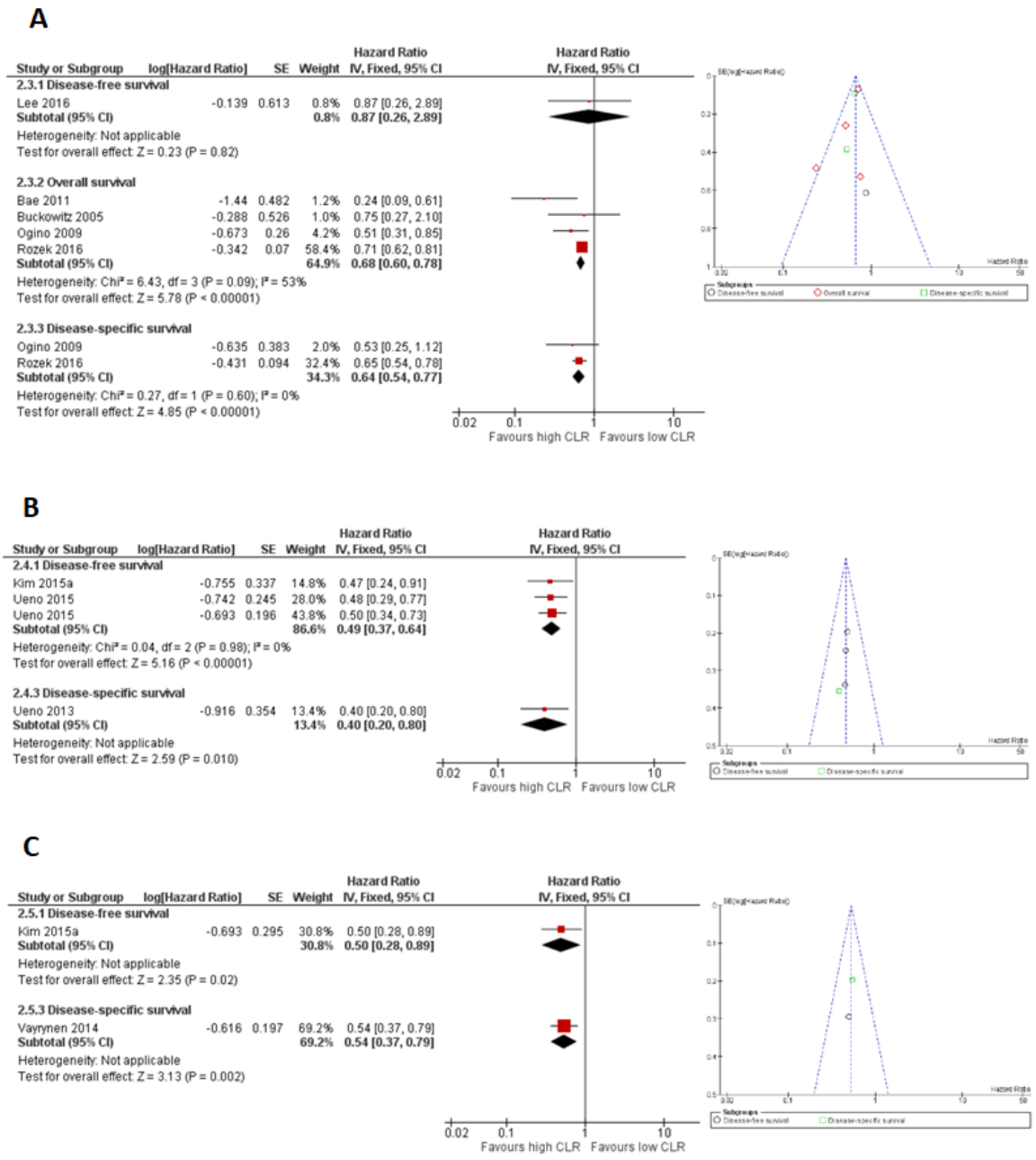


Figure 1.3. Forest plot and funnel plot for CLR in colorectal cancer according to DFS, OS and DSS. A) G-A method, B) Ueno method, C) Väyrynen method

1.5.3.3 H&E Assessment of Tumour Infiltrating Lymphocytes (TILs)

Many groups refer to an assessment of TILs. However, some of these groups, on scrutinizing methodology, are referring instead to a Jass/KM method or similar H&E assessment(Hamada et al., 2018). Therefore, only studies that described counting lymphocytes on H&E-stained slides in an intratumoural compartment (stromal, intraepithelial or both) were considered for this section.

There were nine studies (5508 patients) describing lymphocyte counts in an intratumoural compartment on H&E-stained slides: of which eight (5343 patients) demonstrated a longer survival with higher TILs counts(Turner et al., 2016, Ogino et al., 2009, Ropponen et al., 1997, Lee et al., 2016, Iseki et al., 2018, Nielsen et al., 1999, Rozek et al., 2016, Lang-Schwarz et al., 2018), whereas one study found no survival association(Climent et al., 2019). Four studies reported a semiquantitative assessment of TILs(Turner et al., 2016, Ogino et al., 2009, Ropponen et al., 1997, Lee et al., 2016), while the other 5 counted individual lymphocytes and then dichotomised the data at a set data threshold(Climent et al., 2019, Iseki et al., 2018, Nielsen et al., 1999, Rozek et al., 2016, Lang-Schwarz et al., 2018). None of these studies reported any assessment of interobserver agreement.

Six of these studies assessed MSI status, of which: two studies found TILs to be independent of MSI status for survival(Ogino et al., 2009, Rozek et al., 2016); one found no association between MSI and survival(Turner et al., 2016); whereas 3 studies did not include MSI status in survival analysis(Climent et al., 2019, Lee et al., 2016, Iseki et al., 2018).

Those studies meeting inclusion criteria for meta-analysis of TILs on H&E are given in Tables 1.4 and 1.5 (Figure 1.4). There was one study in colon cancer, which found TILs to be significant for longer DFS and OS (HRs 0.37, 95% CI 0.15-0.90 and 0.45, 95% CI 0.23-0.87, respectively)(Turner et al., 2016). There were six studies in colorectal cancer giving combined fixed effects HRs of 0.65 (95% CI 0.51-0.83) for DFS in 3 studies(Climent et al., 2019, Lee et al., 2016, Ropponen et al., 1997); 0.73 (95% CI 0.64-0.84) for OS in 4

studies(Climent et al., 2019, Nielsen et al., 1999, Ogino et al., 2009, Rozek et al., 2016); and 0.66 (95% CI 0.55-0.78) for DSS in 3 studies(Ogino et al., 2009, Ropponen et al., 1997, Rozek et al., 2016), with no significant heterogeneity and no evidence of publication bias, although numbers were small.

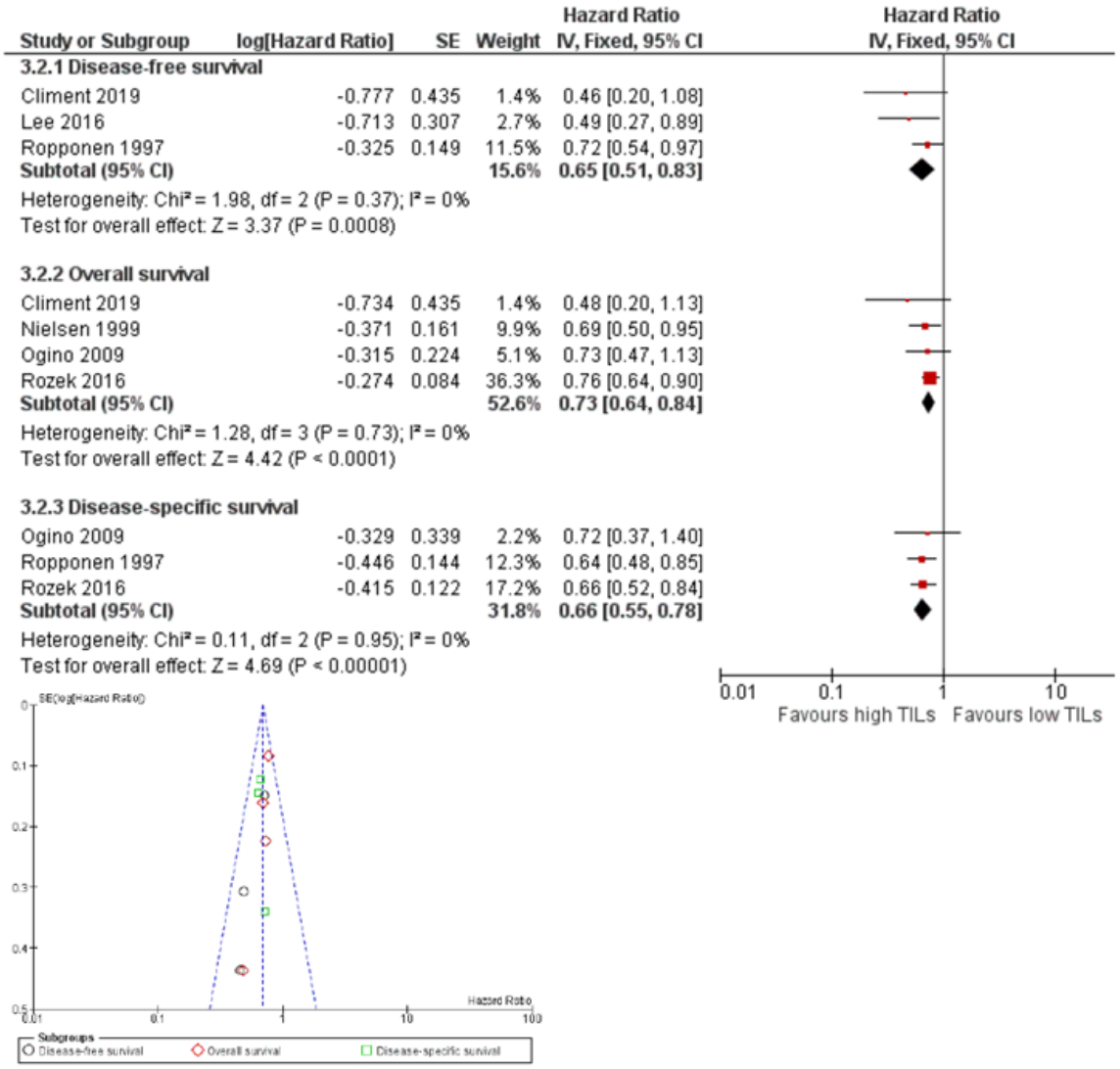


Figure 1.4. Forest plot and funnel plot for TILs in H&E in colorectal cancer according to DFS, OS and DSS

1.5.3.4 Combined H&E Assessment of peritumoural inflammation

Ogino et al.(Ogino et al., 2009) combined KM, CLR and TILs counted separately in stroma and intraepithelial compartments into a score for 843 patients. They assigned a semiquantitative score for each element and then added the individual scores together. Individually, as described above, they found that the separate elements of the score were significant for survival apart from TILs in the intraepithelial compartment. When the scores were combined and arbitrary thresholds were set to separate the score into three groups, the score remained significant for survival and was independent of MSI(Ogino et al., 2009).

Another group replicated the score comparing Caucasian (n=159) and Afro-American (n=52) populations but found that it was only significant in the Afro-American population, independent of MSI(Wallace et al., 2018).

Due to lack of follow up data for the second study, only the original study by Ogino et al.(Ogino et al., 2009) met inclusion criteria for meta-analysis of this score in CRC, significant for OS and DSS (HRs 0.50, 95% CI 0.31-0.81 and 0.31, 95% CI 0.15-0.65, respectively).

1.5.3.5 H&E Assessment and MSI

Overall, 16 studies assessing local inflammatory response on H&E-stained slides also assessed MSI status. Of these, however, 6 did not include MSI in multivariate analysis(Menon et al., 2004, Climent et al., 2019, Gao et al., 2005, Lee et al., 2016, Iseki et al., 2018, Ueno et al., 2013). Of those that did, 5 found MSI not to have any association with survival(Turner et al., 2016, Xie et al., 2018, Chiba et al., 2004, Väyrynen et al., 2014, Buckowitz et al., 2005) and the remaining 5 (3820 patients) found H&E assessment of the local inflammatory response to be independent of MSI for longer survival(Hynes et al., 2017, Ogino et al., 2009, Rozek et al., 2016, Kim et al., 2015a, Wallace et al., 2018).

1.5.4 Immunohistochemistry for Inflammatory Cell Markers

Thus far, a variety of H&E-based methods for assessing the local inflammatory response have been discussed. Some, like KM, assess the inflammatory response in general, whereas others assess more specifically the adaptive immune response, CLR and TILs for example. A range of immunohistochemical stains to specifically select out cell populations have been used and the most common of these are discussed below.

There were 86 studies that stained specifically for T-cell associated markers and tested for associations between these and survival in colon, rectal and colorectal cancer (Table 1.1). However, several studies had overlapping cohorts assessing multiple markers(Bae et al., 2011, Nagtegaal et al., 2001, Kim et al., 2015b, Teng et al., 2015a, McCoy et al., 2015, Sinicrope et al., 2009, Lavotshkin et al., 2015, Chen et al., 2016, Peng et al., 2010, Wang et al., 2018, Galon et al., 2006, Anitei et al., 2014a, Tosolini et al., 2011a, Zlobec et al., 2008b, Zlobec et al., 2008c, Hanke et al., 2015, Koelzer et al., 2014, Baker et al., 2007, Ling et al., 2014, Canna et al., 2005, Zeestraten et al., 2013, Naito et al., 1998). Sixty-three of these cohorts of 14,700 patients were deemed to be independent(Menon et al., 2004, Prizment et al., 2017, Kasajima et al., 2010, Chiba et al., 2004, Teng et al., 2015b, Guidoboni et al., 2001, Flaherty et al., 2016, Miller et al., 2017, Laghi et al., 2009, Deschoolmeester et al., 2010, Katz et al., 2013, Väyrynen et al., 2016, Schweiger et al., 2016, Kim et al., 2018, Eriksen et al., 2018, Lackner et al., 2004, Takemoto et al., 2004, Baeten et al., 2006, Vlad et al., 2015, Li et al., 2018a, Berntsson et al., 2017, Katz et al., 2009, Simpson et al., 2010, Dahlin et al., 2011, Algars et al., 2012, Väyrynen et al., 2012, Richards et al., 2014, Nearchou et al., 2019, Shinto et al., 2014, Posselt et al., 2016, McCoy et al., 2017, Ogura et al., 2018, Chen et al., 2019b, Schollbach et al., 2019, Rosenbaum et al., 2016, Yoon et al., 2012, Prall et al., 2004, Pagès et al., 2009, Suzuki et al., 2010, Makkai-Popa et al., 2013, Mori et al., 2015, Wei et al., 2018, Hu et al., 2018, Loddenkemper et al., 2006, Oshikiri et al., 2006, Salama et al., 2009, Nosho et al., 2010, Lee et al., 2013, Sideras et al., 2018, Nazemalhosseini-Mojarad et

al., 2019, Oberg et al., 2002, Funada et al., 2003, Lugli et al., 2009, Matsutani et al., 2018, Chen and Chen, 2014, Lee et al., 2010, Reimers et al., 2014, Markl et al., 2017, Xu et al., 2013, Wang et al., 2015b, Frey et al., 2010, Wang et al., 2015a, Correale et al., 2010). Of all the studies, 67 found higher densities of T-cells to be associated with longer survival, although only 47 were deemed to assess independent cohorts (13014 patients)(Menon et al., 2004, Prizment et al., 2017, Kasajima et al., 2010, Chiba et al., 2004, Teng et al., 2015b, Guidoboni et al., 2001, Flaherty et al., 2016, Miller et al., 2017, Laghi et al., 2009, Katz et al., 2013, Väyrynen et al., 2016, Kim et al., 2018, Eriksen et al., 2018, Baeten et al., 2006, Vlad et al., 2015, Berntsson et al., 2017, Katz et al., 2009, Simpson et al., 2010, Dahlin et al., 2011, Väyrynen et al., 2012, Richards et al., 2014, Nearchou et al., 2019, Shinto et al., 2014, Posselt et al., 2016, Ogura et al., 2018, Chen et al., 2019b, Schollbach et al., 2019, Yoon et al., 2012, Prall et al., 2004, Pagès et al., 2009, Mori et al., 2015, Hu et al., 2018, Oshikiri et al., 2006, Salama et al., 2009, Nosho et al., 2010, Lee et al., 2013, Nazemalhosseini-Mojarad et al., 2019, Oberg et al., 2002, Funada et al., 2003, Lugli et al., 2009, Matsutani et al., 2018, Lee et al., 2010, Reimers et al., 2014, Markl et al., 2017, Xu et al., 2013, Frey et al., 2010, Wang et al., 2015a). FoxP3 was associated with worse survival outcome in 2 studies(Xu et al., 2013, McCoy et al., 2015), although one of these found both positive and negative survival effects(Xu et al., 2013). No survival association was demonstrated in 18 studies for T-cell densities, of which 14 were deemed to study independent cohorts(Schweiger et al., 2016, Lackner et al., 2004, Takemoto et al., 2004, Li et al., 2018a, Algars et al., 2012, McCoy et al., 2017, Rosenbaum et al., 2016, Suzuki et al., 2010, Makkai-Popa et al., 2013, Wei et al., 2018, Loddenkemper et al., 2006, Sideras et al., 2018, Chen and Chen, 2014, Correale et al., 2010). In many studies, multiple inflammatory cell markers were used in the same multivariate analysis(Nagtegaal et al., 2001, Peng et al., 2010, Flaherty et al., 2016, Väyrynen et al., 2016, Kim et al., 2018, Vlad et al., 2015, Berntsson et al., 2017, Richards et al., 2014, Chen et al., 2019b, Salama et al., 2009, Nosho

et al., 2010, Lee et al., 2013, Lee et al., 2010, Xu et al., 2013) with or without MSI(Baker et al., 2007, Deschoolmeester et al., 2010, Väyrynen et al., 2016, Berntsson et al., 2017, Dahlin et al., 2011, Frey et al., 2010). In this scenario, often each marker on univariate analysis was highly significant for survival, whereas on multivariate analysis of multiple inflammatory markers only one or two markers remained significant. This would suggest that inflammatory cells are not independent of one another, but function in a dependent manner and the survival advantage of a more dense inflammatory cell subset functions only in the context of the cross-talk between multiple inflammatory cells, innate and adaptive, that exist within a functioning immune system(Bonomo et al., 2020).

1.5.4.1 Immunohistochemical Staining for CD3

CD3 (or cluster of differentiation 3) is a protein that is expressed on the cell membrane of all T-cells that have reached maturity and is found in almost no other cell type apart from certain B-cell lymphomas(Lee et al., 2017a). T-cells continue to express CD3 even after further differentiating into cytotoxic (CD8), helper (CD4) or memory (CD45RO) T-cells. As an immunohistochemical marker, therefore, CD3 stains all T-cells and is a good indicator of overall T-cell response(Chetty and Gatter, 1994). There were 34 studies that stained specifically for CD3 in the context of survival in colon, rectal and colorectal cancer, although two studies had overlapping cohorts(Lavotshkin et al., 2015, Peng et al., 2010). There remained, therefore, 32 studies of 7947 patients(Nagtegaal et al., 2001, Sinicrope et al., 2009, Chen et al., 2016, Galon et al., 2006, Hanke et al., 2015, Teng et al., 2015b, Guidoboni et al., 2001, Flaherty et al., 2016, Miller et al., 2017, Laghi et al., 2009, Deschoolmeester et al., 2010, Katz et al., 2013, Väyrynen et al., 2016, Schweiger et al., 2016, Kim et al., 2018, Eriksen et al., 2018, Lackner et al., 2004, Takemoto et al., 2004, Baeten et al., 2006, Vlad et al., 2015, Li et al., 2018a, Berntsson et al., 2017, Katz et al., 2009, Simpson et al., 2010, Dahlin et al., 2011, Algars et al., 2012, Väyrynen et al., 2012, Richards et al., 2014, Nearchou et al., 2019, McCoy et al., 2017, Nosho et al., 2010, Lee et al., 2010). Of these, 23 studies

(5292 patients) found higher densities of CD3 expressing cells to be significant for longer survival(Nagtegaal et al., 2001, Sinicrope et al., 2009, Chen et al., 2016, Galon et al., 2006, Teng et al., 2015b, Guidoboni et al., 2001, Flaherty et al., 2016, Miller et al., 2017, Laghi et al., 2009, Deschoolmeester et al., 2010, Väyrynen et al., 2016, Kim et al., 2018, Eriksen et al., 2018, Baeten et al., 2006, Vlad et al., 2015, Berntsson et al., 2017, Katz et al., 2009, Simpson et al., 2010, Dahlin et al., 2011, Väyrynen et al., 2012, Richards et al., 2014, Nearchou et al., 2019, Lee et al., 2010), whilst the other 9 found no significant difference(Hanke et al., 2015, Katz et al., 2013, Schweiger et al., 2016, Lackner et al., 2004, Takemoto et al., 2004, Li et al., 2018a, Algars et al., 2012, McCoy et al., 2017, Nosho et al., 2010).

There were two studies (229 patients) in rectal cancer assessing the survival impact of CD3 infiltration(Teng et al., 2015b, McCoy et al., 2017). Both studies assessed pre-treatment biopsy specimens for the presence of intratumoural CD3 positive cells. Furthermore, both studies used median values as the data threshold, separating CD3 into equal groups with high and low values. Only one of these (136 patients) found higher CD3 to be significant for improved survival, using a manual counting method(Teng et al., 2015b). The other study employed an electronic counting method but found no significant survival benefit(McCoy et al., 2017). MSI was not assessed by either study. No studies in rectal cancer met inclusion criteria for meta-analysis.

There were six studies (591 patients) in colon cancer assessing the survival impact of CD3 infiltration(Sinicrope et al., 2009, Peng et al., 2010, Guidoboni et al., 2001, Flaherty et al., 2016, Miller et al., 2017, Lee et al., 2010) of which all six demonstrated a longer survival with higher CD3 positive infiltrate. Five studies assessed the density of intratumoural CD3 positive cells(Sinicrope et al., 2009, Peng et al., 2010, Guidoboni et al., 2001, Miller et al., 2017, Lee et al., 2010). Two studies assessed CD3 density at the invasive margin, in conjunction with other tumour areas(Flaherty et al., 2016, Miller et al., 2017). Three studies

assessed and compared the survival impact of CD3 positive cells in more than one tumour compartment in the same cohort: of these, one study found a significantly longer survival for intraepithelial CD3, whilst there was no survival advantage of stromal CD3(Sinicrope et al., 2009); one study found that both CD3 density at the invasive margin and the combined tumour compartments (Total slide) were significant for survival(Flaherty et al., 2016); and one study found that only intratumoural CD3 density was significant for survival, but not at the invasive margin(Miller et al., 2017). There were 3 studies that assessed the presence of MSI: of which two found that CD3 density was significant for survival independent of MSI status(Sinicrope et al., 2009, Guidoboni et al., 2001) and one did not find MSI to be significant for survival(Miller et al., 2017). Four studies assessed CD3 density using an electronic method(Guidoboni et al., 2001, Flaherty et al., 2016, Miller et al., 2017, Lee et al., 2010), two used a manual method(Sinicrope et al., 2009, Peng et al., 2010). Five studies used arbitrary data thresholds(Sinicrope et al., 2009, Peng et al., 2010, Guidoboni et al., 2001, Flaherty et al., 2016, Lee et al., 2010) whilst one study's threshold was data-driven(Miller et al., 2017).

Those studies meeting inclusion criteria for CD3 and survival in colon cancer are given in Table 1.4 (Figure 1.5A&B). There were three studies assessing intratumoural (IT) CD3 in colon cancer giving combined effects HRs of 0.59 (95% CI 0.38-0.91) for DFS in 2 studies(Guidoboni et al., 2001, Sinicrope et al., 2009); 0.49 (95% CI 0.33-0.71) for OS in 3 studies(Guidoboni et al., 2001, Miller et al., 2017, Sinicrope et al., 2009); and one study(Miller et al., 2017) significant for longer DSS (HRs 0.35, 95% CI 0.14-0.88), with no significant heterogeneity and no evidence of publication bias on funnel plot, although numbers are small. There was one study assessing CD3 at the invasive margin (IM), which found no significant survival benefit from a higher density for OS or DSS (HRs 0.48, 95% CI 0.22-1.03 and 1.43, 95% CI 0.31-6.60, respectively).

In colorectal cancer, there were 26 studies comprising 7230 patients assessing the relation of CD3 infiltration with survival(Nagtegaal et al., 2001, Laghi et al., 2009, Deschoolmeester et al., 2010, Katz et al., 2013, Chen et al., 2016, Väyrynen et al., 2016, Schweiger et al., 2016, Kim et al., 2018, Eriksen et al., 2018, Lackner et al., 2004, Takemoto et al., 2004, Baeten et al., 2006, Nosho et al., 2010, Hanke et al., 2015, Vlad et al., 2015, Li et al., 2018a, Berntsson et al., 2017, Katz et al., 2009, Simpson et al., 2010, Dahlin et al., 2011, Algars et al., 2012, Väyrynen et al., 2012, Richards et al., 2014, Nearchou et al., 2019, Lavotshkin et al., 2015, Galon et al., 2006). Seventeen of these (4633 patients) found a higher CD3 density to be associated with longer survival(Nagtegaal et al., 2001, Galon et al., 2006, Laghi et al., 2009, Deschoolmeester et al., 2010, Chen et al., 2016, Väyrynen et al., 2016, Kim et al., 2018, Eriksen et al., 2018, Baeten et al., 2006, Vlad et al., 2015, Berntsson et al., 2017, Katz et al., 2009, Simpson et al., 2010, Dahlin et al., 2011, Väyrynen et al., 2012, Richards et al., 2014, Nearchou et al., 2019), compared with 9 studies (2597 patients) finding no significant survival difference(Katz et al., 2013, Lavotshkin et al., 2015, Schweiger et al., 2016, Lackner et al., 2004, Takemoto et al., 2004, Nosho et al., 2010, Hanke et al., 2015, Algars et al., 2012). Nineteen studies (5414 patients) assessed CD3 in the intratumoural compartments(Nagtegaal et al., 2001, Deschoolmeester et al., 2010, Katz et al., 2013, Chen et al., 2016, Väyrynen et al., 2016, Schweiger et al., 2016, Kim et al., 2018, Eriksen et al., 2018, Lackner et al., 2004, Takemoto et al., 2004, Baeten et al., 2006, Hanke et al., 2015, Vlad et al., 2015, Li et al., 2018a, Berntsson et al., 2017, Katz et al., 2009, Simpson et al., 2010, Algars et al., 2012, Richards et al., 2014) and of these 12 studies (3579 patients) found a significant association with longer survival(Nagtegaal et al., 2001, Deschoolmeester et al., 2010, Chen et al., 2016, Väyrynen et al., 2016, Kim et al., 2018, Eriksen et al., 2018, Baeten et al., 2006, Vlad et al., 2015, Berntsson et al., 2017, Katz et al., 2009, Simpson et al., 2010, Richards et al., 2014). Nine studies (1641 patients) assessed CD3 and the invasive margin(Nagtegaal et al., 2001, Laghi et al., 2009, Deschoolmeester et al., 2010, Väyrynen et

al., 2016, Schweiger et al., 2016, Lackner et al., 2004, Baeten et al., 2006, Li et al., 2018a, Richards et al., 2014), of which 4 studies (775 patients) were significant for longer survival(Nagtegaal et al., 2001, Laghi et al., 2009, Väyrynen et al., 2016, Richards et al., 2014). Six studies (1668 patients) assessed CD3 on the whole slide(Nosho et al., 2010, Dahlin et al., 2011, Väyrynen et al., 2012, Nearchou et al., 2019, Lavotshkin et al., 2015, Galon et al., 2006) of which four (906 patients) were significant for survival(Dahlin et al., 2011, Väyrynen et al., 2012, Nearchou et al., 2019, Galon et al., 2006). Seven studies significant for survival assessed more than one tumour region and of these: three found that IE CD3 and not ST CD3 was significant for survival(Deschoolmeester et al., 2010, Väyrynen et al., 2016, Simpson et al., 2010), whereas three found that both IE CD3 and ST CD3 were significant for survival(Kim et al., 2018, Baeten et al., 2006, Richards et al., 2014); two studies found that intratumoural assessment of CD3 was significant whereas assessment at the invasive margin was not(Deschoolmeester et al., 2010, Baeten et al., 2006), while three found that assessment of both intratumoural and invasive margin CD3 was significant for survival(Nagtegaal et al., 2001, Väyrynen et al., 2016, Richards et al., 2014). Nine studies assessed the presence of MSI: of these two studies found CD3 to be independent of MSI(Laghi et al., 2009, Väyrynen et al., 2016); two studies found no association of MSI with survival and therefore it was not included in multivariate analysis(Eriksen et al., 2018, Takemoto et al., 2004); a further two studies did not include MSI in survival analysis(Kim et al., 2018, Simpson et al., 2010); and in 3 studies CD3 was not independent of MSI on multivariate analysis(Deschoolmeester et al., 2010, Berntsson et al., 2017, Dahlin et al., 2011). Fifteen studies used an electronic method of assessment(Laghi et al., 2009, Katz et al., 2013, Chen et al., 2016, Väyrynen et al., 2016, Kim et al., 2018, Eriksen et al., 2018, Nosho et al., 2010, Vlad et al., 2015, Li et al., 2018a, Berntsson et al., 2017, Katz et al., 2009, Väyrynen et al., 2012, Nearchou et al., 2019, Lavotshkin et al., 2015, Galon et al., 2006) of which 11 found CD3 to be significant for survival(Laghi et al., 2009, Chen et al., 2016,

Väyrynen et al., 2016, Kim et al., 2018, Eriksen et al., 2018, Vlad et al., 2015, Berntsson et al., 2017, Katz et al., 2009, Väyrynen et al., 2012, Nearchou et al., 2019, Galon et al., 2006). Twelve studies used a manual assessment method(Nagtegaal et al., 2001, Deschoolmeester et al., 2010, Schweiger et al., 2016, Lackner et al., 2004, Takemoto et al., 2004, Baeten et al., 2006, Hanke et al., 2015, Simpson et al., 2010, Dahlin et al., 2011, Algars et al., 2012, Väyrynen et al., 2012, Richards et al., 2014), of which 7 found CD3 to be associated with survival(Nagtegaal et al., 2001, Deschoolmeester et al., 2010, Baeten et al., 2006, Simpson et al., 2010, Dahlin et al., 2011, Väyrynen et al., 2012, Richards et al., 2014). The only study to directly compare electronic and manual methods of CD3 assessment in colorectal cancer found both to be comparable and significant for survival(Väyrynen et al., 2012). Thirteen studies used various different arbitrary data thresholds(Deschoolmeester et al., 2010, Schweiger et al., 2016, Kim et al., 2018, Eriksen et al., 2018, Takemoto et al., 2004, Baeten et al., 2006, Nosho et al., 2010, Hanke et al., 2015, Vlad et al., 2015, Simpson et al., 2010, Dahlin et al., 2011, Algars et al., 2012, Richards et al., 2014), whereas 9 studies used data-driven thresholds(Laghi et al., 2009, Katz et al., 2013, Chen et al., 2016, Väyrynen et al., 2016, Berntsson et al., 2017, Katz et al., 2009, Väyrynen et al., 2012, Nearchou et al., 2019, Galon et al., 2006). In four studies, the method of threshold determination was unclear(Nagtegaal et al., 2001, Lackner et al., 2004, Li et al., 2018a, Lavotshkin et al., 2015).

Those studies meeting inclusion criteria for meta-analysis of CD3 are given in Table 1.5 (Figure 1.5C&D). Twelve studies met inclusion criteria for meta-analysis of IT CD3 in colorectal cancer, giving combined effects HRs of: 0.46 (95% CI 0.39-0.54) for DFS in six studies(Chen et al., 2016, Deschoolmeester et al., 2010, Eriksen et al., 2018, Galon et al., 2006, Kim et al., 2018, Väyrynen et al., 2016), (moderate heterogeneity, I^2 56%; p=0.04); 0.57 (95% CI 0.50-0.64) for OS in eight studies(Berntsson et al., 2017, Chen et al., 2016, Deschoolmeester et al., 2010, Eriksen et al., 2018, Galon et al., 2006, Kim et al., 2018, Nosho et al., 2010, Väyrynen et al., 2016), (substantial heterogeneity (I^2 73%; p<0.001); and 0.59

(95% CI 0.50-0.70) for DSS in six studies(Dahlin et al., 2011, Nosho et al., 2010, Richards et al., 2014, Simpson et al., 2010, Väyrynen et al., 2012, Väyrynen et al., 2016), (no significant heterogeneity). The funnel plot did not suggest any evidence of publication bias. Eight studies were included for IM CD3 in colorectal cancer giving combined effects HRs of: 0.45 (95% CI 0.33-0.61) for DFS in three studies(Deschoolmeester et al., 2010, Galon et al., 2006, Väyrynen et al., 2016), (no significant heterogeneity); 0.71 (95% CI 0.59-0.85) for OS in four studies(Deschoolmeester et al., 2010, Galon et al., 2006, Nosho et al., 2010, Väyrynen et al., 2016), (substantial heterogeneity (I^2 67%; p=0.03); and 0.58 (95% CI 0.48-0.69) for DSS in six studies(Dahlin et al., 2011, Laghi et al., 2009, Nosho et al., 2010, Richards et al., 2014, Väyrynen et al., 2012, Väyrynen et al., 2016), (no significant heterogeneity). Funnel plot did not suggest any evidence of publication bias.

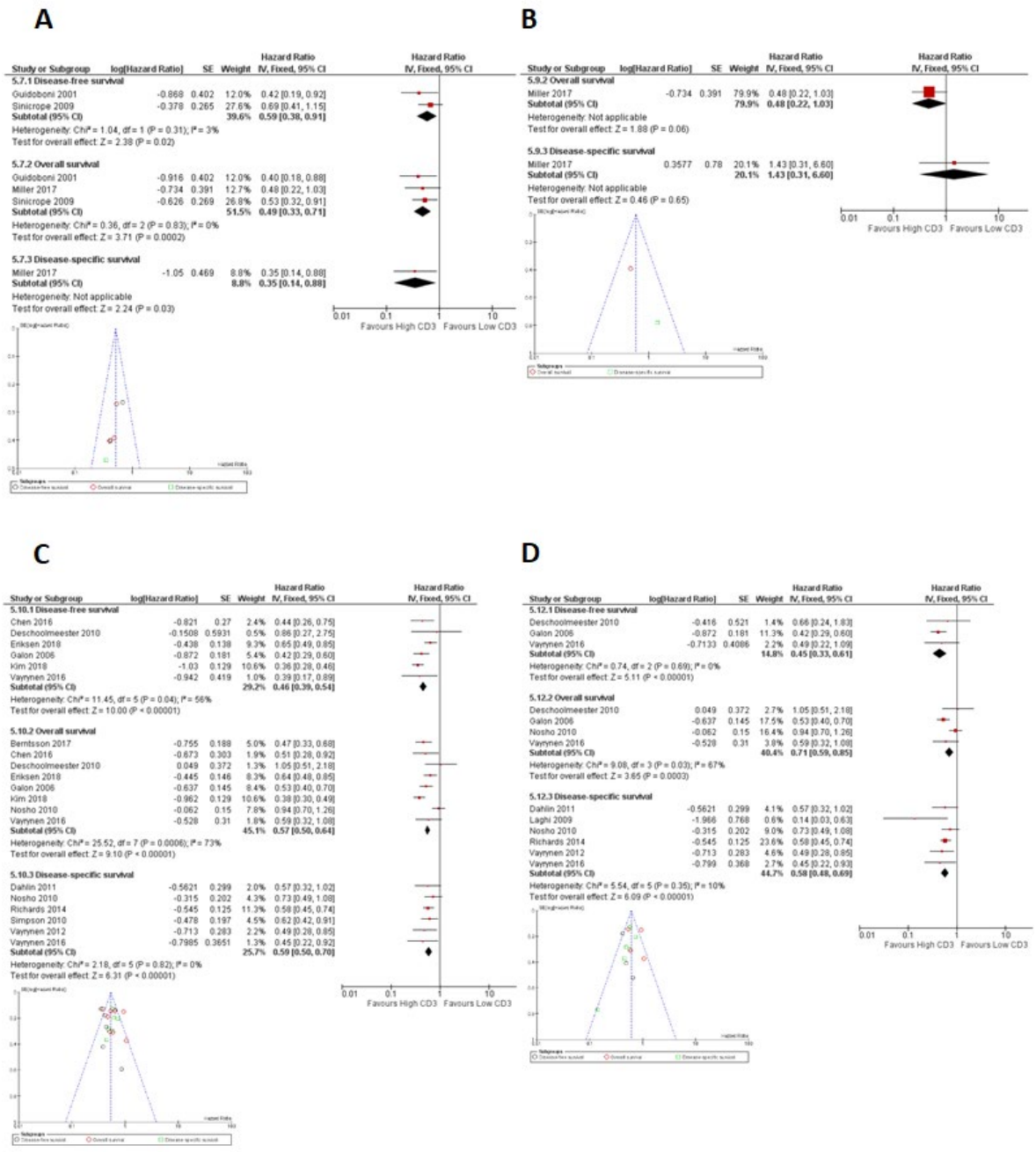


Figure 1.5. Forest plots and funnel plots for CD3 according to DFS, OS and DSS: A) IT in colon cancer; B) IM in colon cancer; C) IT in colorectal cancer; D) IM in colorectal cancer

1.5.4.2 Immunohistochemical Staining for CD8

CD8 (cluster of differentiation 8) is a protein that is expressed on the cell surface of cytotoxic T-cells, whose role is to recognise a foreign antigen presented by an antigen presenting cell and to bind cells expressing that antigen, inducing cell lysis and recruiting other immune cells with the release of cytokines(Bakshi et al., 2014). CD8 was assessed in 62 studies in relation to survival in rectal, colon or colorectal cancer, although there were twelve studies whose cohorts overlapped(Anitei et al., 2014b, Teng et al., 2015a, Koelzer et al., 2014, Tosolini et al., 2011b, Kim et al., 2015b, Lavotshkin et al., 2015, Chen et al., 2016, Naito et al., 1998, Bae et al., 2011, Hanke et al., 2015, Zlobec et al., 2008a, Zlobec et al., 2008c). There remained, therefore, 50 studies assessing CD8 in independent cohorts comprising a total of 12868 patients(Teng et al., 2015b, Guidoboni et al., 2001, Flaherty et al., 2016, Miller et al., 2017, Deschoolmeester et al., 2010, Katz et al., 2013, Väyrynen et al., 2016, Schweiger et al., 2016, Kim et al., 2018, Eriksen et al., 2018, Takemoto et al., 2004, Baeten et al., 2006, Li et al., 2018a, Berntsson et al., 2017, Katz et al., 2009, Richards et al., 2014, Shinto et al., 2014, Posselt et al., 2016, McCoy et al., 2017, Ogura et al., 2018, Chen et al., 2019b, Schollbach et al., 2019, Rosenbaum et al., 2016, Yoon et al., 2012, Prall et al., 2004, Menon et al., 2004, Pagès et al., 2009, Suzuki et al., 2010, Makkai-Popa et al., 2013, Mori et al., 2015, Wei et al., 2018, Hu et al., 2018, Loddenkemper et al., 2006, Oshikiri et al., 2006, Salama et al., 2009, Kasajima et al., 2010, Nosho et al., 2010, Lee et al., 2013, Prizment et al., 2017, Sideras et al., 2018, Nazemalhosseini-Mojarad et al., 2019, Nagtegaal et al., 2001, Ling et al., 2014, Oberg et al., 2002, Funada et al., 2003, Chiba et al., 2004, Lugli et al., 2009, Matsutani et al., 2018, Correale et al., 2010, Baker et al., 2007). Of these, CD8 was found to be significant for longer survival in 37 studies(Teng et al., 2015b, Guidoboni et al., 2001, Flaherty et al., 2016, Deschoolmeester et al., 2010, Katz et al., 2013, Väyrynen et al., 2016, Kim et al., 2018, Eriksen et al., 2018, Berntsson et al., 2017, Katz et al., 2009, Richards et al., 2014, Shinto et al., 2014, Posselt et al., 2016, Ogura et al., 2018,

Chen et al., 2019b, Schollbach et al., 2019, Yoon et al., 2012, Prall et al., 2004, Menon et al., 2004, Pagès et al., 2009, Mori et al., 2015, Hu et al., 2018, Oshikiri et al., 2006, Salama et al., 2009, Kasajima et al., 2010, Nosho et al., 2010, Lee et al., 2013, Prizment et al., 2017, Nazemalhosseini-Mojarad et al., 2019, Baker et al., 2007, Nagtegaal et al., 2001, Ling et al., 2014, Oberg et al., 2002, Funada et al., 2003, Chiba et al., 2004, Lugli et al., 2009, Matsutani et al., 2018) comprising 11085 patients, whilst no significant association between CD8 and survival was found in 13 studies(Rosenbaum et al., 2016, McCoy et al., 2017, Correale et al., 2010, Miller et al., 2017, Suzuki et al., 2010, Makkai-Popa et al., 2013, Schweiger et al., 2016, Wei et al., 2018, Takemoto et al., 2004, Loddenkemper et al., 2006, Baeten et al., 2006, Li et al., 2018a, Sideras et al., 2018).

In rectal cancer, there were eleven independent studies (1749 patients) assessing CD8 and survival(Shinto et al., 2014, Anitei et al., 2014b, Teng et al., 2015b, Posselt et al., 2016, McCoy et al., 2017, Ogura et al., 2018, Chen et al., 2019b, Schollbach et al., 2019, Zlobec et al., 2008a, Koelzer et al., 2014, Rosenbaum et al., 2016). Nine of these (1463 patients) found higher CD8 density to be significant for longer survival(Shinto et al., 2014, Anitei et al., 2014b, Teng et al., 2015b, Posselt et al., 2016, Ogura et al., 2018, Chen et al., 2019b, Schollbach et al., 2019, Zlobec et al., 2008a, Koelzer et al., 2014). Seven studies (949 patients) assessed CD8 in intratumoural compartments on pre-treatment biopsies (Shinto et al., 2014, Teng et al., 2015b, Posselt et al., 2016, McCoy et al., 2017, Ogura et al., 2018, Chen et al., 2019b, Koelzer et al., 2014). Of these, three found (497 patients) a significant association of CD8 in pre-treatment biopsies with survival (Teng et al., 2015b, Ogura et al., 2018, Koelzer et al., 2014). While six studies (1269 patients) assessed CD8 in the intratumoural compartment of resected specimens(Shinto et al., 2014, Posselt et al., 2016, Ogura et al., 2018, Chen et al., 2019b, Zlobec et al., 2008a, Rosenbaum et al., 2016), of which four studies (804 patients) were significantly associated with longer survival(Shinto et al., 2014, Posselt et al., 2016, Chen et al., 2019b, Zlobec et al., 2008a). Two studies

assessed the combined invasive margin and intratumoural compartments for CD8 infiltration (162 patients), both of which were significant for survival(Anitei et al., 2014b, Schollbach et al., 2019). Four studies assessed CD8 infiltration in biopsies taken prior to neo-adjuvant therapy, in addition to post-resection specimens: of these, three studies (346 patients) found that CD8 levels in the resected specimen were significantly associated with survival while those in the biopsies were not(Shinto et al., 2014, Posselt et al., 2016, Chen et al., 2019b); whereas in 1 study (285 patients) found that CD8 in the biopsy associated with survival, but not in the resected specimen(Ogura et al., 2018). Only one study in full resection specimens compared more than one tumour compartment, finding ST CD8 to be significantly associated with survival, where IE CD8 was not(Posselt et al., 2016). Three studies used an electronic method of assessment(Anitei et al., 2014b, Posselt et al., 2016, McCoy et al., 2017), of which 2 studies found CD8 to be significant for survival(Anitei et al., 2014b, Posselt et al., 2016). Eight studies used a manual assessment method(Shinto et al., 2014, Teng et al., 2015b, Ogura et al., 2018, Chen et al., 2019b, Schollbach et al., 2019, Zlobec et al., 2008a, Koelzer et al., 2014, Rosenbaum et al., 2016), of which 7 found CD8 to be significant for survival(Shinto et al., 2014, Teng et al., 2015b, Ogura et al., 2018, Chen et al., 2019b, Schollbach et al., 2019, Zlobec et al., 2008a, Koelzer et al., 2014). Three studies assessed the presence of MSI: of which 1 did not find MSI to be independently significant for survival(Rosenbaum et al., 2016); whereas the other 2 did not include MSI in the survival analysis(Zlobec et al., 2008a, Koelzer et al., 2014). Nine studies used an arbitrary threshold(Shinto et al., 2014, Teng et al., 2015b, Posselt et al., 2016, McCoy et al., 2017, Ogura et al., 2018, Chen et al., 2019b, Schollbach et al., 2019, Koelzer et al., 2014, Rosenbaum et al., 2016), of which 7 found CD8 to be significant for survival(Shinto et al., 2014, Teng et al., 2015b, Posselt et al., 2016, Ogura et al., 2018, Chen et al., 2019b, Schollbach et al., 2019, Koelzer et al., 2014). Two studies used a data-driven threshold, both of which were significant for survival(Anitei et al., 2014b, Zlobec et al., 2008a).

Those studies meeting inclusion criteria for meta-analysis of CD8 in rectal cancer are given in Table 1.6 (Figure 1.6A). Three studies met inclusion criteria for meta-analysis of IT CD8. There was one study assessing DFS and OS, which found higher CD8 density to be significant for longer DFS, but not OS (HRs 0.38, 95% CI 0.14-0.99 and 0.44, 95% CI 0.13-1.53, respectively) (Chen et al., 2019b). Two studies assessed DSS, giving a combined effects HR of: 0.52 (95% CI 0.39-0.69), with substantial heterogeneity (I^2 73%; p=0.05)(Rosenbaum et al., 2016, Zlobec et al., 2008a). Funnel plot could not be interpreted due since there were too few studies. No studies met inclusion criteria for meta-analysis of IM CD8 in rectal cancer.

Table 1.6. Meta-analysis results for studies assessing survival (DFS, OS and DSS) in rectal cancer in relation to peritumoural inflammatory assessments

Impact of study methodology on heterogeneity testing (I^2 test) and overall effect							
		Overall effect			Heterogeneity		
Location assessed	**Survival type**	**No. of studies**	**HR**	**95% CI**	**I^2 test (%)**	***P*-value**	**First Author Surname/year**
CD8							
IT	DFS	1	0.38	0.14-0.99	NA		Chen 19
	OS	1	0.44	0.13-1.53	NA		Chen 19
	DSS	2	0.52	0.39-0.69	73	0.05	Rosenbaum, Zlobec 08a
FoxP3							
IT	DFS	1	0.72	0.56-0.93	NA		Reimers
	OS	1	0.73	0.56-0.95	NA		Reimers
CD56/57							
IT	OS	1	0.23	0.08-0.66	NA		Alderdice

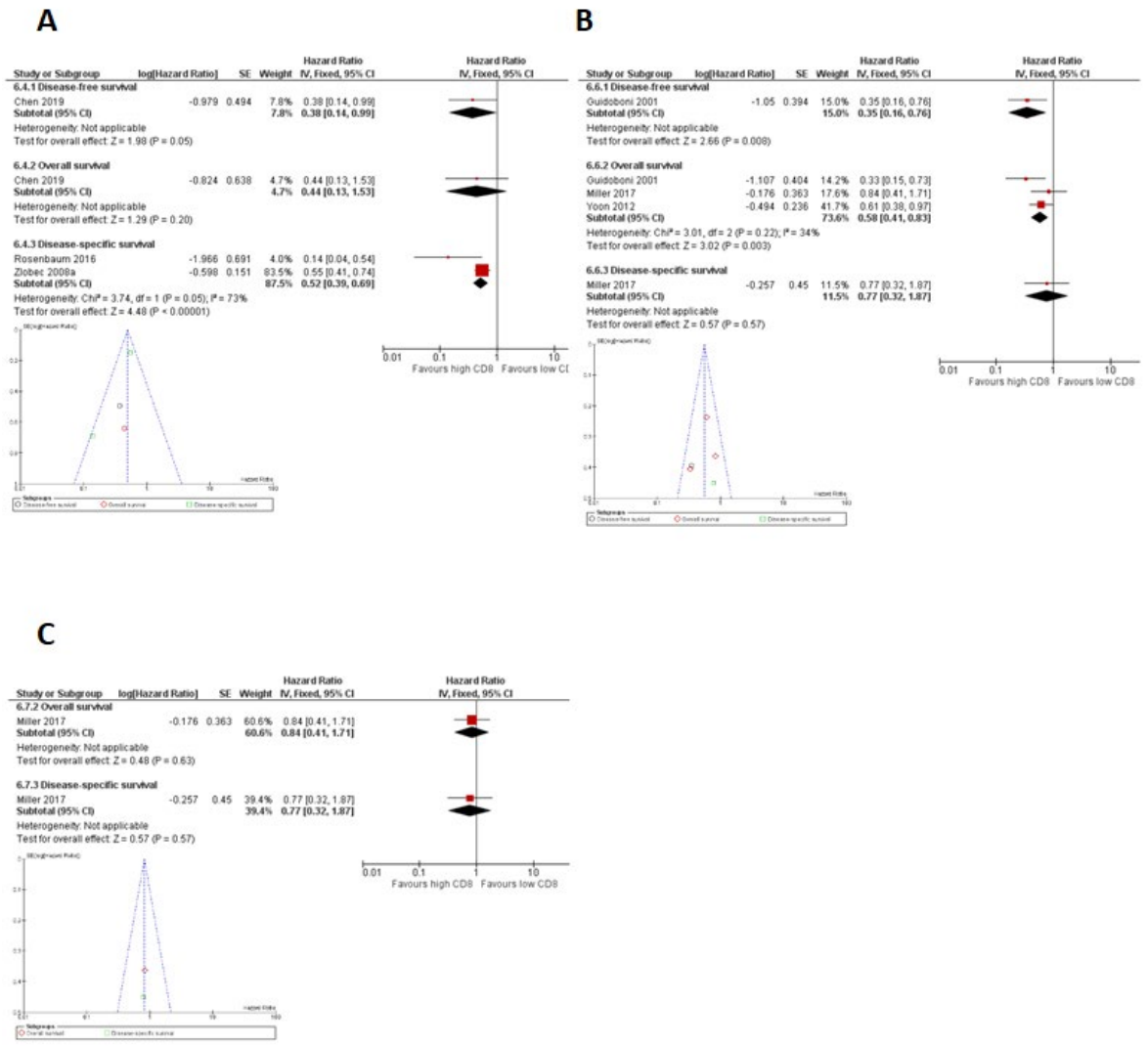


Figure 1.6. Forest plots and funnel plots for CD8 according to DFS, OS and DSS: A) IT in rectal cancer; B) IT in colon cancer; C) IM in colon cancer

In colon cancer, there were six independent studies (811 patients) assessing CD8 and survival: of which four (650 patients) found it to be significantly associated with longer survival(Guidoboni et al., 2001, Zlobec et al., 2008c, Flaherty et al., 2016, Yoon et al., 2012), whereas 2 did not(Correale et al., 2010, Miller et al., 2017), although one of these was in stage IV disease(Correale et al., 2010). All the studies assessing CD8 in colon cancer assessed the intratumoural compartment. The only study assessing both IE CD8 and ST CD8 separately found both to be significant for survival(Yoon et al., 2012). Two studies (193 patients) assessed CD8 at the invasive margin(Flaherty et al., 2016, Miller et al., 2017), of which only one (89 patients) was significant for survival(Flaherty et al., 2016). Only one study (89 patients) assessed CD8 on the whole slide, finding it to be significant for survival(Flaherty et al., 2016). This study was also the only study with significant results comparing intratumoural and invasive margin CD8, finding both to be significant for survival(Flaherty et al., 2016). Three studies used an electronic method of assessment(Guidoboni et al., 2001, Flaherty et al., 2016, Miller et al., 2017), of which two found CD8 to be significant for survival(Guidoboni et al., 2001, Flaherty et al., 2016). Three studies used a manual method of assessment(Zlobec et al., 2008c, Correale et al., 2010, Yoon et al., 2012), of which 2 found CD8 to be significant for survival(Zlobec et al., 2008c, Yoon et al., 2012). Four studies assessed the presence of MSI: of which 1 found IE CD8 to be independent of MSI(Guidoboni et al., 2001); one found ST CD8, but not IE CD8 to be independent of MSI(Yoon et al., 2012); one found that MSI was not significant for survival(Miller et al., 2017); and one excluded all MSI patients from survival analysis(Zlobec et al., 2008c). Four studies used an arbitrary data threshold for analysis(Guidoboni et al., 2001, Correale et al., 2010, Flaherty et al., 2016, Yoon et al., 2012), of which 3 were significant for survival(Guidoboni et al., 2001, Flaherty et al., 2016, Yoon et al., 2012). Two studies used a data-driven threshold(Zlobec et al., 2008c, Miller et al., 2017), of which one was significant for survival(Zlobec et al., 2008c).

Those studies meeting inclusion criteria for meta-analysis of CD8 in colon cancer are given in Table 1.4 (Figure 1.6B&C). Three studies met inclusion criteria for meta-analysis for IT CD8 in colon cancer: one study assessed DFS, finding higher CD8 density to be significant for longer survival (HR 0.35, 95% CI 0.16-0.76),(Guidoboni et al., 2001); three studies assessed OS, giving a combined effects HR of: 0.58 (95% CI 0.41-0.83), with moderate heterogeneity (I^2 34%; p=0.22),(Guidoboni et al., 2001, Miller et al., 2017, Yoon et al., 2012); while one assessed DSS, finding no significant difference in survival (HR 0.77, 95% CI 0.32-1.87),(Miller et al., 2017). Funnel plot could not be assessed for publication bias due to small numbers. One study was included for IM CD8, which did not identify any significant association with OS or DSS (HRs 0.84, 95% CI 0.41-1.71 and 0.77, 95% CI 0.32-1.87, respectively),(Miller et al., 2017).

In colorectal cancer, there were 44 studies assessing CD8, 38 of which (11274 patients) assessed independent cohorts(Nagtegaal et al., 2001, Baker et al., 2007, Prall et al., 2004, Menon et al., 2004, Pagès et al., 2009, Suzuki et al., 2010, Deschoolmeester et al., 2010, Makkai-Popa et al., 2013, Lavotshkin et al., 2015, Mori et al., 2015, Väyrynen et al., 2016, Schweiger et al., 2016, Wei et al., 2018, Hu et al., 2018, Kim et al., 2018, Eriksen et al., 2018, Takemoto et al., 2004, Loddenkemper et al., 2006, Baeten et al., 2006, Oshikiri et al., 2006, Salama et al., 2009, Kasajima et al., 2010, Nosho et al., 2010, Lee et al., 2013, Li et al., 2018a, Prizment et al., 2017, Berntsson et al., 2017, Sideras et al., 2018, Nazemalhosseini-Mojarad et al., 2019, Oberg et al., 2002, Funada et al., 2003, Chiba et al., 2004, Lugli et al., 2009, Katz et al., 2013, Katz et al., 2009, Richards et al., 2014, Ling et al., 2014, Matsutani et al., 2018). Twenty-six of these (9903 patients) found CD8 to be significant for longer survival(Nagtegaal et al., 2001, Baker et al., 2007, Prall et al., 2004, Menon et al., 2004, Pagès et al., 2009, Deschoolmeester et al., 2010, Mori et al., 2015, Väyrynen et al., 2016, Hu et al., 2018, Kim et al., 2018, Eriksen et al., 2018, Oshikiri et al., 2006, Salama et al., 2009, Kasajima et al., 2010, Nosho et al., 2010, Lee et al., 2013,

Prizment et al., 2017, Berntsson et al., 2017, Nazemalhosseini-Mojarad et al., 2019, Oberg et al., 2002, Funada et al., 2003, Chiba et al., 2004, Lugli et al., 2009, Katz et al., 2013, Katz et al., 2009, Richards et al., 2014, Ling et al., 2014, Matsutani et al., 2018). Thirty-four independent studies (10168 patients) assessed intratumoural CD8(Nagtegaal et al., 2001, Lee et al., 2013, Prall et al., 2004, Menon et al., 2004, Pagès et al., 2009, Suzuki et al., 2010, Deschoolmeester et al., 2010, Makkai-Popa et al., 2013, Mori et al., 2015, Väyrynen et al., 2016, Schweiger et al., 2016, Wei et al., 2018, Hu et al., 2018, Kim et al., 2018, Eriksen et al., 2018, Takemoto et al., 2004, Loddenkemper et al., 2006, Baeten et al., 2006, Oshikiri et al., 2006, Salama et al., 2009, Kasajima et al., 2010, Li et al., 2018a, Prizment et al., 2017, Berntsson et al., 2017, Sideras et al., 2018, Nazemalhosseini-Mojarad et al., 2019, Oberg et al., 2002, Chiba et al., 2004, Baker et al., 2007, Katz et al., 2013, Katz et al., 2009, Richards et al., 2014, Ling et al., 2014, Matsutani et al., 2018), of which 25 (8736 patients) were associated with longer survival(Nagtegaal et al., 2001, Prall et al., 2004, Pagès et al., 2008, Deschoolmeester et al., 2010, Mori et al., 2015, Väyrynen et al., 2016, Hu et al., 2018, Kim et al., 2018, Eriksen et al., 2018, Oshikiri et al., 2006, Salama et al., 2009, Kasajima et al., 2010, Prizment et al., 2017, Berntsson et al., 2017, Nazemalhosseini-Mojarad et al., 2019, Oberg et al., 2002, Chiba et al., 2004, Baker et al., 2007, Katz et al., 2013, Katz et al., 2009, Richards et al., 2014, Ling et al., 2014, Matsutani et al., 2018). Of the nine studies that directly compared CD8 assessment in different intratumoural compartments: three studies (625 patients) found IE significant where ST was not(Nazemalhosseini-Mojarad et al., 2019, Naito et al., 1998, Deschoolmeester et al., 2010); two studies (460 patients) found ST significant where IE was not(Väyrynen et al., 2016, Matsutani et al., 2018); three studies (1294 patients) found both IE and ST to be significant(Kim et al., 2018, Richards et al., 2014, Ling et al., 2014); and one (291 patients) study compared IE assessment with combined assessment of IE and ST and found IE alone to be significant(Kasajima et al., 2010). There were 17 independent studies (4027 patients) that assessed CD8 at the invasive

margin(Nagtegaal et al., 2001, Menon et al., 2004, Pagès et al., 2009, Deschoolmeester et al., 2010, Makkai-Popa et al., 2013, Kim et al., 2015b, Väyrynen et al., 2016, Schweiger et al., 2016, Naito et al., 1998, Baeten et al., 2006, Li et al., 2018a, Sideras et al., 2018, Funada et al., 2003, Lugli et al., 2009, Richards et al., 2014, Ling et al., 2014, Matsutani et al., 2018), of which eight (2491 patients) found IM CD8 to be significant for survival(Menon et al., 2004, Pagès et al., 2009, Kim et al., 2015b, Funada et al., 2003, Lugli et al., 2009, Richards et al., 2014, Ling et al., 2014, Matsutani et al., 2018). Ten studies (2271 patients) compared CD8 at the IM and in the intratumoural compartment: of which only one (96 patients) found IM CD8 to be significant for survival where intratumoural assessments were not(Menon et al., 2004); four studies (691 patients) found intratumoural CD8 significant where IM CD8 was not(Naito et al., 1998, Deschoolmeester et al., 2010, Väyrynen et al., 2016, Nagtegaal et al., 2001); five studies (1484 patients) found both intratumoural and IM CD8 to be significant for survival(Pagès et al., 2009, Kim et al., 2015b, Richards et al., 2014, Ling et al., 2014, Matsutani et al., 2018). Four independent studies compared CD8 on the whole slide (combined IM and intratumoural): of which three (1335 patients) were significant for survival(Pagès et al., 2009, Nosho et al., 2010, Ling et al., 2014); and one was not significant, but this study was small with only 35 patients(Lavotshkin et al., 2015). Fifteen independent studies (5475 patients) used an electronic method of assessment(Pagès et al., 2009, Makkai-Popa et al., 2013, Lavotshkin et al., 2015, Väyrynen et al., 2016, Kim et al., 2018, Eriksen et al., 2018, Salama et al., 2009, Nosho et al., 2010, Lee et al., 2013, Li et al., 2018a, Prizment et al., 2017, Berntsson et al., 2017, Sideras et al., 2018, Katz et al., 2009, Katz et al., 2013), of which 11 (4949 patients) found CD8 to be significant for survival(Pagès et al., 2009, Väyrynen et al., 2016, Kim et al., 2018, Eriksen et al., 2018, Salama et al., 2009, Nosho et al., 2010, Lee et al., 2013, Prizment et al., 2017, Berntsson et al., 2017, Katz et al., 2009, Katz et al., 2013). Twenty-two independent studies (5534 patients) used a manual method of assessment(Nagtegaal et al., 2001, Prall et al., 2004, Menon et al., 2004, Suzuki et al.,

2010, Deschoolmeester et al., 2010, Mori et al., 2015, Schweiger et al., 2016, Wei et al., 2018, Hu et al., 2018, Takemoto et al., 2004, Loddenkemper et al., 2006, Baeten et al., 2006, Oshikiri et al., 2006, Kasajima et al., 2010, Nazemalhosseini-Mojarad et al., 2019, Oberg et al., 2002, Funada et al., 2003, Chiba et al., 2004, Baker et al., 2007, Richards et al., 2014, Ling et al., 2014, Matsutani et al., 2018), of which 16 (4689 patients) found CD8 to be significant for survival(Nagtegaal et al., 2001, Prall et al., 2004, Menon et al., 2004, Deschoolmeester et al., 2010, Mori et al., 2015, Hu et al., 2018, Oshikiri et al., 2006, Kasajima et al., 2010, Nazemalhosseini-Mojarad et al., 2019, Oberg et al., 2002, Funada et al., 2003, Chiba et al., 2004, Baker et al., 2007, Richards et al., 2014, Ling et al., 2014, Matsutani et al., 2018). Eighteen independent studies assessed the presence of MSI: of which 4 found CD8 to be independent of MSI(Nazemalhosseini-Mojarad et al., 2019, Prall et al., 2004, Ling et al., 2014, Matsutani et al., 2018); five found MSI to be not significant for survival(Salama et al., 2009, Wei et al., 2018, Eriksen et al., 2018, Takemoto et al., 2004, Chiba et al., 2004); three found that CD8 was not independent of MSI on multivariate analysis(Deschoolmeester et al., 2010, Väyrynen et al., 2016, Berntsson et al., 2017); and six did not include MSI in multivariate analysis(Menon et al., 2004, Mori et al., 2015, Hu et al., 2018, Kim et al., 2018, Prizment et al., 2017, Lugli et al., 2009). Twenty-five studies (6853 patients) used an arbitrary data threshold(Prall et al., 2004, Menon et al., 2004, Suzuki et al., 2010, Deschoolmeester et al., 2010, Makkai-Popa et al., 2013, Mori et al., 2015, Schweiger et al., 2016, Wei et al., 2018, Kim et al., 2018, Eriksen et al., 2018, Takemoto et al., 2004, Loddenkemper et al., 2006, Baeten et al., 2006, Salama et al., 2009, Kasajima et al., 2010, Nosho et al., 2010, Lee et al., 2013, Prizment et al., 2017, Sideras et al., 2018, Nazemalhosseini-Mojarad et al., 2019, Funada et al., 2003, Chiba et al., 2004, Richards et al., 2014, Ling et al., 2014, Matsutani et al., 2018), of which 17 (5936 patients) found CD8 to be significant for survival(Prall et al., 2004, Menon et al., 2004, Deschoolmeester et al., 2010, Mori et al., 2015, Kim et al., 2018, Eriksen et al., 2018, Salama et al., 2009, Kasajima

et al., 2010, Nosho et al., 2010, Lee et al., 2013, Prizment et al., 2017, Nazemalhosseini-Mojarad et al., 2019, Funada et al., 2003, Chiba et al., 2004, Richards et al., 2014, Ling et al., 2014, Matsutani et al., 2018). Seven studies used a data-driven threshold, all of which found CD8 to be significant for survival(Pagès et al., 2009, Väyrynen et al., 2016, Hu et al., 2018, Berntsson et al., 2017, Lugli et al., 2009, Katz et al., 2013, Katz et al., 2009). The threshold method was unclear for 5 studies(Nagtegaal et al., 2001, Lavotshkin et al., 2015, Li et al., 2018a, Oberg et al., 2002, Oshikiri et al., 2006).

Those studies meeting inclusion criteria for meta-analysis of CD8 in colorectal cancer are given in Table 1.5 (Figure 1.7). Twenty-two studies met inclusion criteria for meta-analysis of IT CD8 in colorectal cancer, giving combined effects HRs of: 0.46 (95% CI 0.39-0.54) for DFS in eight studies(Chen et al., 2016, Deschoolmeester et al., 2010, Eriksen et al., 2018, Kim et al., 2018, Mori et al., 2015, Prall et al., 2004, Tosolini et al., 2011b, Väyrynen et al., 2016), (moderate heterogeneity, I^2 48%; p=0.06); 0.63 (95% CI 0.58-0.67) for OS in fifteen studies(Berntsson et al., 2017, Chen et al., 2016, Deschoolmeester et al., 2010, Eriksen et al., 2018, Kasajima et al., 2010, Kim et al., 2018, Naito et al., 1998, Nazemalhosseini-Mojarad et al., 2019, Nosho et al., 2010, Oshikiri et al., 2006, Pagès et al., 2009, Prizment et al., 2017, Salama et al., 2009, Väyrynen et al., 2016, Zlobec et al., 2008b), (substantial heterogeneity, I^2 65%; p<0.001); and 0.62 (95% CI 0.56-0.69) for DSS in nine studies(Baker et al., 2007, Chiba et al., 2004, Ling et al., 2014, Nosho et al., 2010, Pagès et al., 2009, Prall et al., 2004, Prizment et al., 2017, Richards et al., 2014, Väyrynen et al., 2016), (substantial heterogeneity, I^2 73%; p<0.001). The funnel plot revealed no significant publication bias, although one study in particular was seen as an outlier for DFS, OS and DSS(Pagès et al., 2009).

Eight studies met inclusion criteria for meta-analysis of IM CD8 in colorectal cancer, giving combined effects HRs of: 0.50 (95% CI 0.40-0.62) for DFS in four studies(Deschoolmeester et al., 2010, Kim et al., 2015b, Tosolini et al., 2011b, Väyrynen et al., 2016), (no significant heterogeneity); 0.62 (95% CI 0.51-0.75) for OS in four studies(Deschoolmeester et al., 2010,

Kim et al., 2015b, Nosho et al., 2010, Väyrynen et al., 2016), (substantial heterogeneity, I^2 58%; p=0.05); and 0.53 (95% CI 0.45-0.63) for DSS in five studies(Lugli et al., 2009, Matsutani et al., 2018, Nosho et al., 2010, Richards et al., 2014, Väyrynen et al., 2016), (substantial heterogeneity, I^2 53%; p=0.05). The funnel plot did not suggest any significant publication bias. There is one small left-sided outlier skewing results and heterogeneity for DSS, although curiously only for one of the two populations in the same study(Matsutani et al., 2018). Exclusion of this outlying population resulted in no significant heterogeneity for DSS, data not shown.

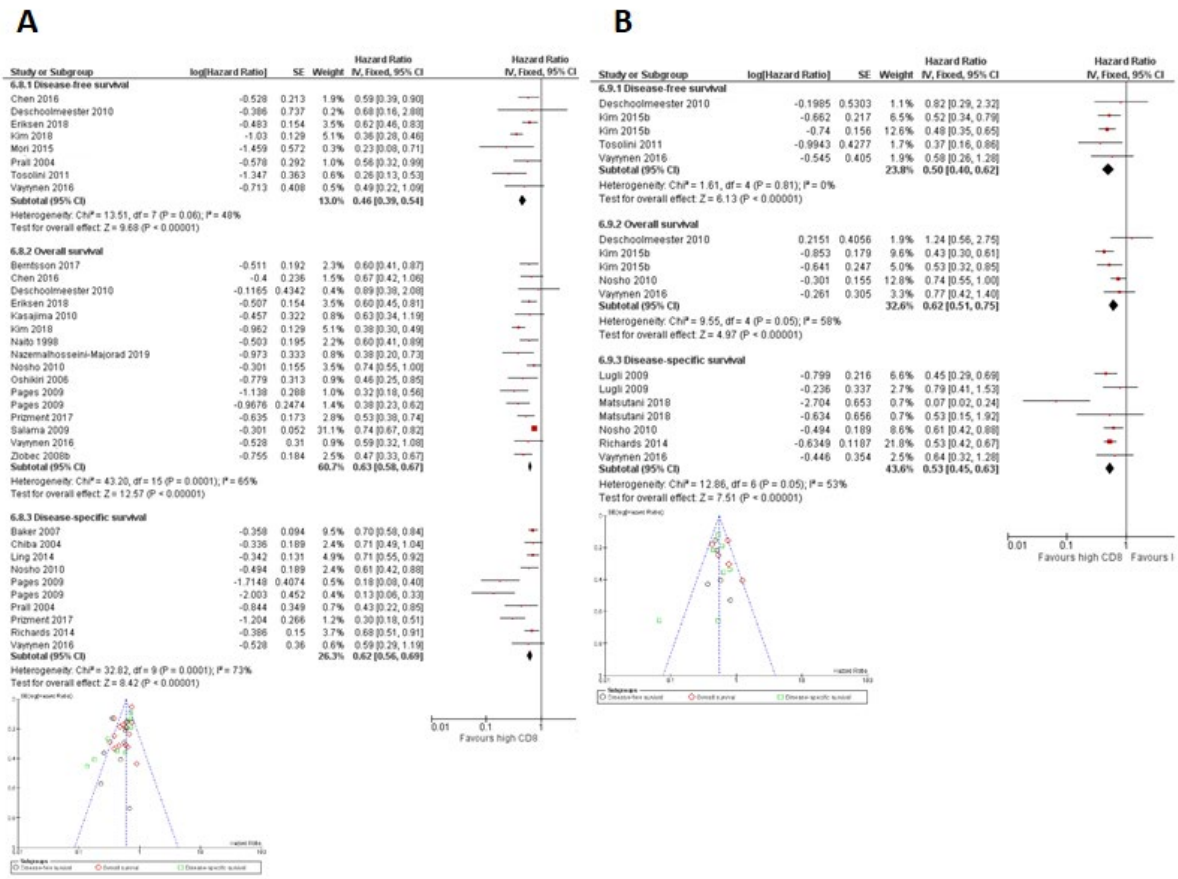


Figure 1.7. Forest plots and funnel plots for CD8 according to DFS, OS and DSS: A) IT in colorectal cancer; B) IM in colorectal cancer

1.5.4.3 Immunohistochemical Staining for CD4

CD4 (cluster of differentiation 4) is a protein that is expressed on the cell surface of helper T-cells, , although it is expressed to a lesser degree by other immune cells and therefore is not entirely helper T-cell specific(Mak and Saunders, 2006). CD4 T-cells play a considerable role in anti-cancer immunity with both cytotoxic capabilities and roles in recruitment and priming both cytotoxic T-cells and recruiting B-cells to the tumour microenvironment(Borst et al., 2018). There were fifteen studies on CD4 and rectal or colorectal cancer survival, but the cohort in one overlapped with another(Wei et al., 2018), leaving fourteen independent studies comprising 2726 patients(Teng et al., 2015a, Nagtegaal et al., 2001, Menon et al., 2004, Katz et al., 2013, Katz et al., 2009, Makkai-Popa et al., 2013, Lavotshkin et al., 2015, Chen et al., 2016, Kasajima et al., 2010, Chen and Chen, 2014, Li et al., 2018a, Canna et al., 2005, Ling et al., 2014, Matsutani et al., 2018). One of these (62 patients) assessed survival in rectal cancer but did not meet inclusion criteria for meta-analysis. They assessed pre-treatment biopsies but did not find IT CD4 to be significant for survival(Teng et al., 2015a).

The remaining thirteen independent studies (2664 patients) assessed CD4 in colorectal cancer: of which six studies (1471 patients) found CD4 to be significantly associated with longer survival(Katz et al., 2013, Katz et al., 2009, Chen et al., 2016, Kasajima et al., 2010, Canna et al., 2005, Ling et al., 2014); while the 7 remaining studies (1193 patients) found no association between CD4 and survival(Nagtegaal et al., 2001, Menon et al., 2004, Makkai-Popa et al., 2013, Lavotshkin et al., 2015, Chen and Chen, 2014, Li et al., 2018a, Matsutani et al., 2018). Eleven studies assessed intratumoural CD4(Nagtegaal et al., 2001, Menon et al., 2004, Katz et al., 2013, Katz et al., 2009, Makkai-Popa et al., 2013, Chen et al., 2016, Kasajima et al., 2010, Li et al., 2018a, Canna et al., 2005, Ling et al., 2014, Matsutani et al., 2018), of which six found it to be significant for survival(Katz et al., 2013, Katz et al., 2009, Chen et al., 2016, Kasajima et al., 2010, Canna et al., 2005, Ling et al., 2014). None of the 5 studies assessing CD4 at the invasive margin, nor the 2 studies assessing

CD4 on the whole slide found any significant association with survival(Nagtegaal et al., 2001, Menon et al., 2004, Makkai-Popa et al., 2013, Lavotshkin et al., 2015, Chen and Chen, 2014, Li et al., 2018a, Matsutani et al., 2018). There were no studies with significant findings that compared CD4 in different tumour regions. Six studies used an electronic method of assessment(Katz et al., 2013, Katz et al., 2009, Makkai-Popa et al., 2013, Lavotshkin et al., 2015, Li et al., 2018a, Chen et al., 2016), of which 3 found CD4 to be significant for survival(Katz et al., 2013, Katz et al., 2009, Chen et al., 2016). Seven studies used a manual method of CD4 assessment(Nagtegaal et al., 2001, Menon et al., 2004, Kasajima et al., 2010, Canna et al., 2005, Ling et al., 2014, Chen and Chen, 2014, Matsutani et al., 2018), of which 3 found it to be significant for survival(Kasajima et al., 2010, Canna et al., 2005, Ling et al., 2014). Four studies assessed the presence of MSI: of which 2 found CD4 to be independent of MSI for survival(Ling et al., 2014, Matsutani et al., 2018); one did not find MSI to be significant for survival(Wei et al., 2018); and one did not include MSI in multivariate analysis(Menon et al., 2004). Seven studies used an arbitrary data threshold(Menon et al., 2004, Makkai-Popa et al., 2013, Wei et al., 2018, Kasajima et al., 2010, Canna et al., 2005, Ling et al., 2014, Matsutani et al., 2018), of which 3 found CD4 to be significant for survival(Kasajima et al., 2010, Canna et al., 2005, Ling et al., 2014). Three studies used a data-driven threshold, all of which were significant for survival(Katz et al., 2013, Katz et al., 2009, Chen et al., 2016). The data threshold method was unclear for 4 studies(Nagtegaal et al., 2001, Lavotshkin et al., 2015, Chen and Chen, 2014, Li et al., 2018a).

Those studies meeting inclusion criteria for meta-analysis of CD4 in colorectal cancer are given in Table 1.5. Three studies met inclusion criteria for meta-analysis for IT CD4: one assessed DFS finding a significant survival benefit (HR 0.55; 95% CI: 0.32-0.96), (Chen et al., 2016); two assessed OS, giving a combined effects HR of 0.64 (95% CI: 0.42-0.97), (no significant heterogeneity),(Chen et al., 2016, Kasajima et al., 2010); and one assessed DSS finding a significant survival benefit (HR 0.64; 95% CI: 0.41-0.99), (Ling et al., 2014). There

were too few papers to give meaningful results from funnel plot analysis. No studies reporting IM CD4 were included in meta-analysis. There were no studies identified addressing CD4 in colon cancer.

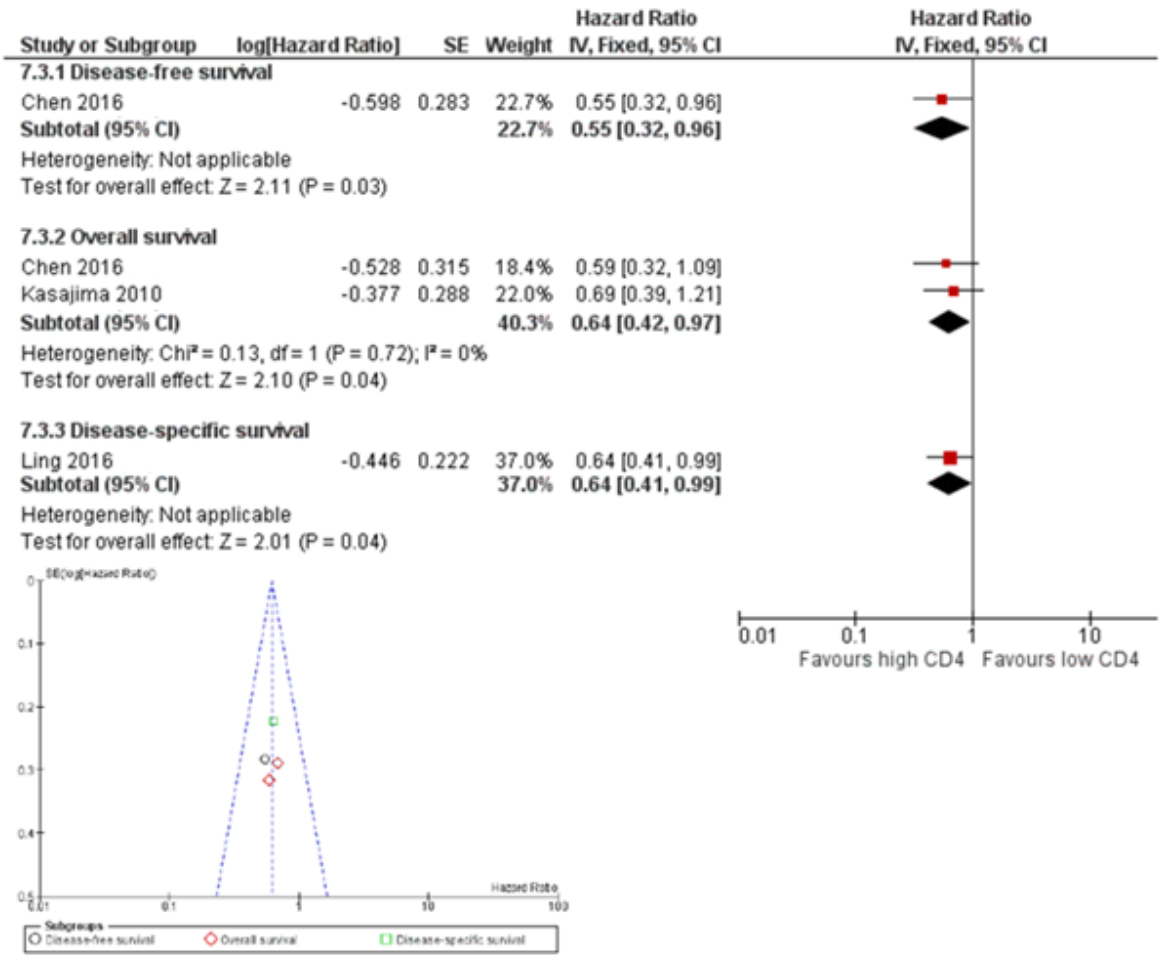


Figure 1.8. Forest plot and funnel plot for IT CD4 according to DFS, OS and DSS in colorectal cancer

1.5.4.4 Immunohistochemical Staining for CD45RO

CD45RO (cluster of differentiation 45, isoform RO) is a protein that is expressed on the cell surface of effector memory T-cells, after they have matured from naïve memory T-cells (isoform RA). The role of the effector memory T-cell is to enact a swift response to a recognised foreign antigen(Sallusto et al., 1999). CD45RO has largely been studied in this context. However, it may also be expressed by B-cells and to a lesser degree by other immune cells and therefore is not entirely specific to effector memory T-cells(Mahnke et al., 2013, Dawes et al., 2006, Zhou and Tedder, 1996).

There were fifteen studies assessing CD45RO and survival in rectal, colon or colorectal cancer, one of which had an overlapping cohort(Peng et al., 2010), leaving 14 independent studies comprising 4235 patients(Kim et al., 2015b, Chen et al., 2016, Koelzer et al., 2014, Schweiger et al., 2016, Katz et al., 2009, Richards et al., 2014, Pagès et al., 2009, Makkai-Popa et al., 2013, Salama et al., 2009, Nosho et al., 2010, Lee et al., 2013, Oberg et al., 2002, Lee et al., 2010, Wang et al., 2015b). Twelve studies found CD45RO to be associated with better survival, but only eleven (3992 patients) of these were independent(Wang et al., 2015b, Koelzer et al., 2014, Lee et al., 2010, Pagès et al., 2009, Kim et al., 2015b, Chen et al., 2016, Salama et al., 2009, Nosho et al., 2010, Lee et al., 2013, Oberg et al., 2002, Richards et al., 2014).

In rectal cancer, there were two studies (263 patients) assessing CD45RO, both finding it to be significantly associated with survival: one of which performed a manual assessment of CD45RO in pre-treatment biopsies(Koelzer et al., 2014), the other an electronic assessment in post-resection specimens(Wang et al., 2015b). Neither study compared CD45RO in more than one tumour region. One assessed the presence of MSI but did not include this in multivariate analysis(Koelzer et al., 2014). Both studies used an arbitrary data threshold. Neither study met inclusion criteria for meta-analysis in rectal cancer.

In colon cancer, there were two studies (166 patients) assessing intratumoural CD45RO, both finding it to be significantly associated with survival: one of which used a manual method(Peng et al., 2010), while the other used an electronic counting method(Lee et al., 2010). Neither study assessed the invasive margin, nor the presence of MSI. Both studies used an arbitrary data threshold. Neither met inclusion criteria for meta-analysis of CD45RO in colon cancer.

In colorectal cancer, there were 11 studies (3885 patients) assessing CD45RO and survival(Pagès et al., 2009, Makkai-Popa et al., 2013, Kim et al., 2015b, Chen et al., 2016, Schweiger et al., 2016, Salama et al., 2009, Nosho et al., 2010, Lee et al., 2013, Oberg et al., 2002, Katz et al., 2009, Richards et al., 2014), of which 8 studies (3642 patients) found a significant association with survival(Pagès et al., 2009, Kim et al., 2015b, Chen et al., 2016, Salama et al., 2009, Nosho et al., 2010, Lee et al., 2013, Oberg et al., 2002, Richards et al., 2014). In those not primarily assessing stage IV disease (3590 patients), 7 out of 8 studies reported longer survival for those with high CD45RO(Pagès et al., 2009, Kim et al., 2015b, Chen et al., 2016, Salama et al., 2009, Nosho et al., 2010, Oberg et al., 2002, Richards et al., 2014), with only 1 small study (27 patients) reporting no significant survival association(Makkai-Popa et al., 2013). Ten studies assessed intratumoural CD45RO(Pagès et al., 2009, Makkai-Popa et al., 2013, Kim et al., 2015b, Chen et al., 2016, Schweiger et al., 2016, Salama et al., 2009, Lee et al., 2013, Oberg et al., 2002, Katz et al., 2009, Richards et al., 2014), of which seven found it to be associated with survival(Pagès et al., 2009, Kim et al., 2015b, Chen et al., 2016, Salama et al., 2009, Lee et al., 2013, Oberg et al., 2002, Richards et al., 2014). The only study comparing CD45RO in more than one intratumoural compartment found both IE and ST CD45RO to be significantly associated with survival(Richards et al., 2014). Five studies assessed CD45RO at the invasive margin(Pagès et al., 2009, Schweiger et al., 2016, Lee et al., 2013, Richards et al., 2014, Kim et al., 2015b), of which 4 found it to be significant for survival(Pagès et al., 2009, Kim et al., 2015b, Lee

et al., 2013, Richards et al., 2014). Of the 4 studies that compared assessment at the invasive margin with intratumoural assessment, all found that both areas were associated with better survival(Pagès et al., 2009, Kim et al., 2015b, Lee et al., 2013, Richards et al., 2014). Two studies performed a combined assessment of the full slide (invasive margin and intratumoural) and both found CD45RO to be significantly associated with survival(Pagès et al., 2009, Nosho et al., 2010). Eight studies used an electronic method of assessment(Pagès et al., 2009, Makkai-Popa et al., 2013, Kim et al., 2015b, Chen et al., 2016, Salama et al., 2009, Nosho et al., 2010, Lee et al., 2013, Katz et al., 2009), of which 6 found CD45RO to be significant for survival(Pagès et al., 2009, Kim et al., 2015b, Chen et al., 2016, Salama et al., 2009, Nosho et al., 2010, Lee et al., 2013). Of the 3 studies using a manual assessment method: two found CD45RO to be significant for survival(Oberg et al., 2002, Richards et al., 2014), whereas one did not(Schweiger et al., 2016). Two studies assessed the presence of MSI, but neither included it in multivariate analysis(Salama et al., 2009, Kim et al., 2015b). Seven studies used an arbitrary data threshold(Makkai-Popa et al., 2013, Kim et al., 2015b, Schweiger et al., 2016, Salama et al., 2009, Nosho et al., 2010, Lee et al., 2013, Richards et al., 2014), of which 5 found CD45RO to be significantly associated with survival(Kim et al., 2015b, Salama et al., 2009, Nosho et al., 2010, Lee et al., 2013, Richards et al., 2014). Three studies used a data-driven threshold(Pagès et al., 2009, Chen et al., 2016, Katz et al., 2009) and 2 of these found CD45RO to be associated with survival(Chen et al., 2016, Pagès et al., 2009). Data threshold method was unclear in one study(Oberg et al., 2002).

Those studies meeting inclusion criteria for meta-analysis of CD45RO in colorectal cancer are given in Table 1.5 (Figure 1.9). There were six studies in colorectal cancer assessing IT CD45RO, giving combined fixed effects HRs of 0.52 (95% CI 0.40-0.69) for DFS in 3 studies(Chen et al., 2016, Kim et al., 2015b, Pagès et al., 2009), (substantial heterogeneity, I^2 83%; p=0.003); 0.53 (95% CI 0.61-0.75) for OS in 5 studies(Chen et al., 2016, Kim et al., 2015b, Nosho et al., 2010, Pagès et al., 2009, Salama et al., 2009), (substantial heterogeneity,

I^2 78%; p=0.001); and 0.53 (95% CI 0. 0.44-0.64) for DSS in 3 studies(Nosho et al., 2010, Pagès et al., 2009, Richards et al., 2014), (substantial heterogeneity, I^2 83%; p=0.002). As with CD8, the IT CD45RO funnel plot revealed no significant publication bias, although one study in particular was seen as an outlier for DFS, OS and DSS(Pagès et al., 2009), skewing results to the left and removal of this study from meta-analysis resulted in a large fall in the heterogeneity to 0%, 51% and 70% for DFS, OS and DSS, respectively (data not shown). There were three studies in colorectal cancer assessing IM CD45RO. One study assessed DFS finding IM CD45RO significant for longer DFS (HR 0.42, 95% CI 0.33-0.54) (Kim et al., 2015b), whilst for OS and DSS combined fixed effects HRs were 0.51 (95% CI 0.42-0.63) for OS in 2 studies(Kim et al., 2015b, Nosho et al., 2010), (substantial heterogeneity, I^2 77%; p=0.01); and 0.57 (95% CI 0.47-0.68) for DSS in 2 studies(Nosho et al., 2010, Richards et al., 2014), (substantial heterogeneity, I^2 70%; p=0.07). Funnel plot contained too few studies to give meaningful data regarding publication bias, although the plot was narrow indicating larger studies with similar results.

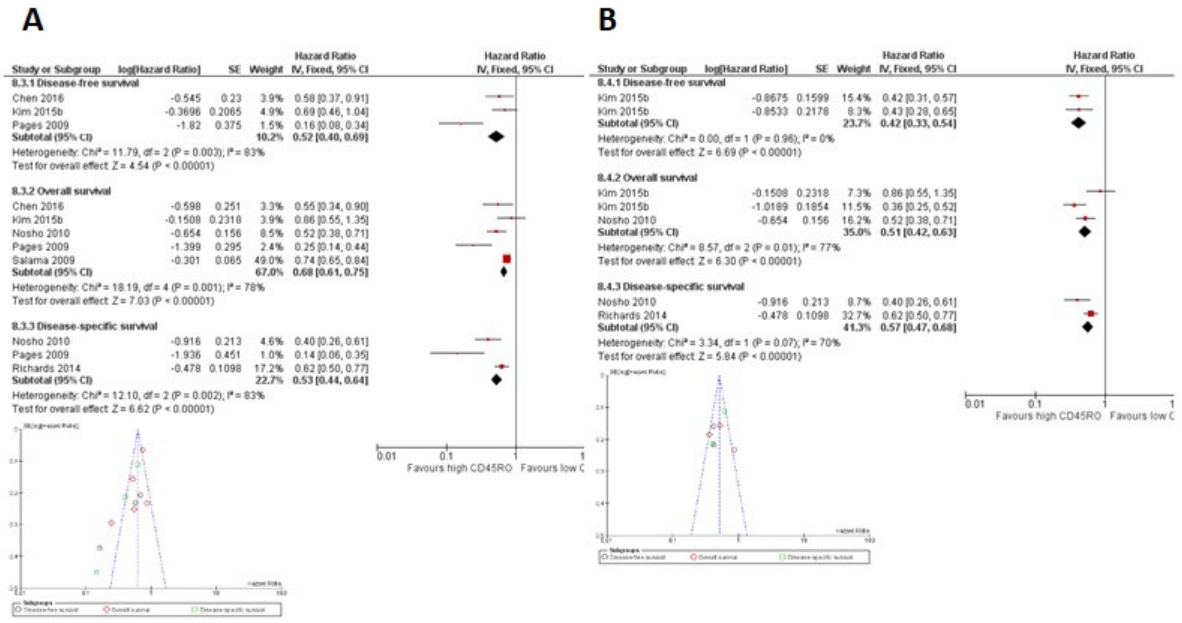


Figure 1.9. Forest plots and funnel plots for CD45RO according to DFS, OS and DSS: A) IT in colorectal cancer; B) IM in colorectal cancer

1.5.4.5 Immunohistochemical Staining for FoxP3

FoxP3 (Forkhead box protein P3) is commonly expressed by regulatory T-cells(Colamatteo et al., 2019). A large body of research has been performed on regulatory T-cells in a variety of cancers as well as non-neoplastic research. Due to the fact that their function is to regulate the immune system by suppressing T-cell activity, thereby preventing overactivity and autoimmunity(Spence et al., 2015), their presence in and around colorectal cancer may be expected to be a poor prognostic indicator as some have hypothesised(Suzuki et al., 2010). Some have gone as far as to say that they may support tumorigenesis(Pastille et al., 2019, Suzuki et al., 2010).

Thirty-four studies were identified that assessed rectal, colon or colorectal cancer prognosis related to FoxP3 expression in TILs, but there were three overlapping cohorts(McCoy et al., 2017, Sinicrope et al., 2009, Hanke et al., 2015), leaving 31 independent studies (7991 patients)(Kim et al., 2015b, Suzuki et al., 2010, Katz et al., 2013, Zeestraten et al., 2013, Lavotshkin et al., 2015, Mori et al., 2015, Schweiger et al., 2016, Loddenkemper et al., 2006, Lee et al., 2013, Chen and Chen, 2014, Sideras et al., 2018, Shinto et al., 2014, McCoy et al., 2015, Chen et al., 2016, Väyrynen et al., 2016, Salama et al., 2009, Nosho et al., 2010, Xu et al., 2013, Vlad et al., 2015, Wang et al., 2015a, Berntsson et al., 2017, Frey et al., 2010, Ling et al., 2014, Richards et al., 2014, Lee et al., 2010, Correale et al., 2010, Yoon et al., 2012, Miller et al., 2017, Markl et al., 2017, Reimers et al., 2014, Posselt et al., 2016). Twenty-one studies found FoxP3 to be associated with longer survival, but only twenty (6774 patients) of these were independent(Kim et al., 2015b, Chen et al., 2016, Väyrynen et al., 2016, Salama et al., 2009, Nosho et al., 2010, Lee et al., 2013, Xu et al., 2013, Vlad et al., 2015, Wang et al., 2015a, Berntsson et al., 2017, Frey et al., 2010, Ling et al., 2014, Richards et al., 2014, Lee et al., 2010, Correale et al., 2010, Yoon et al., 2012, Miller et al., 2017, Markl et al., 2017, Reimers et al., 2014, Posselt et al., 2016), compared with 2 studies (218 patients) finding FoxP3 to have a detrimental impact on survival(Xu et al., 2013,

McCoy et al., 2015), one of which had both positive and negative findings(Xu et al., 2013). Twelve studies found no impact of FoxP3 expression on survival(Suzuki et al., 2010, Katz et al., 2013, Zeestraten et al., 2013, Lavotshkin et al., 2015, Mori et al., 2015, Schweiger et al., 2016, Loddenkemper et al., 2006, Chen and Chen, 2014, Sideras et al., 2018, Sinicrope et al., 2009, Shinto et al., 2014, McCoy et al., 2017).

In rectal cancer, there were four independent studies (840 patients) assessing FoxP3: of which two (631 patients) found a positive association with survival(Reimers et al., 2014, Posselt et al., 2016); one (128 patients) found a negative association with survival(McCoy et al., 2015); and one found no association(Shinto et al., 2014). Three studies assessed FoxP3 in pre-treatment biopsies: of which only one (153 patients) found it to have a significant positive association with survival(Posselt et al., 2016); while the other two found no association(Shinto et al., 2014, McCoy et al., 2017). Four studies assessed the intratumoural compartment of post-resection specimens: of which one study found a significant positive association with survival(Reimers et al., 2014); one found a significant negative association(McCoy et al., 2015); and two found no significant association(Shinto et al., 2014, Posselt et al., 2016). One study assessed FoxP3 in both pre-treatment biopsies and post-resection specimens, finding its presence in the biopsy to be significant for better survival, whereas there was no survival significance of FoxP3 in the resected specimen(Posselt et al., 2016). Of the two studies that assessed the same cohort: one assessed FoxP3 in pre-treatment biopsies, finding no significant association with survival(McCoy et al., 2017), whereas in the resected specimen, there was a significant negative association of the presence of FoxP3 with survival(McCoy et al., 2015). No groups assessed the invasive margin or the whole tumour slide for FoxP3 in rectal cancer. Two independent studies used an electronic method of assessment and a data-driven threshold, of which one (128 patients) found a negative impact of FoxP3 infiltration in the resected tumour on survival(McCoy et al., 2015), whereas one (153 patients) found a positive impact of FoxP3 in the pre-treatment biopsy on

survival(Posselt et al., 2016). Two studies used a manual method of assessment and arbitrary threshold, of which one (81 patients) found no significant impact on survival in pre-treatment biopsy or resected specimen(Shinto et al., 2014), whereas one (478 patients) found a significant positive impact of FoxP3 on the resected specimen(Reimers et al., 2014). No studies assessed the presence of MSI.

Only one study met inclusion criteria for meta-analysis of IT FoxP3 in rectal cancer (Table 1.6, Figure 1.10), finding a significant association with longer DFS and OS (HRs 0.72, 95% CI 0.56-0.93 and 0.73, 95% CI 0.56-0.95, respectively)(Reimers et al., 2014).

In colon cancer, there were five independent studies (540 patients) assessing FoxP3, all of which found a positive association with survival(Lee et al., 2010, Correale et al., 2010, Yoon et al., 2012, Miller et al., 2017, Markl et al., 2017). Four of these assessed the intratumoural compartments(Yoon et al., 2012, Correale et al., 2010, Lee et al., 2010, Miller et al., 2017) and two assessed the invasive margin(Miller et al., 2017, Markl et al., 2017). The only study (104 patients) assessing both intratumoural compartments and the invasive margin found that IT FoxP3 was significant for longer survival, whereas IM FoxP3 was not significant(Miller et al., 2017). However, the other study of IM FoxP3 (136 patients) found that this was significant for longer survival(Markl et al., 2017). None of the studies assessed FoxP3 in the whole slide in colon cancer. Three studies used an electronic method of assessment(Lee et al., 2010, Correale et al., 2010, Miller et al., 2017), whereas two used a manual assessment method(Yoon et al., 2012, Markl et al., 2017). Three studies assessed the presence of MSI: of which one found the presence of FoxP3 to be independent of MSI(Yoon et al., 2012); one found that MSI was not significant for survival(Miller et al., 2017); and one found that FoxP3 was not independent of MSI(Markl et al., 2017). Three studies used arbitrary data thresholds(Lee et al., 2010, Correale et al., 2010, Yoon et al., 2012), while two used data-driven thresholds(Miller et al., 2017, Markl et al., 2017).

Two studies met inclusion criteria for meta-analysis of IT FoxP3 in colon cancer (Table 1.4, Figure 1.10). One study assessed DFS finding no significant survival association (HR 1.23, 95%CI: 0.72-2.13) (Sinicrope et al., 2009); two studies assessed OS with a combined fixed effects HR of 0.91 (95% CI 0.59-1.40), (substantial heterogeneity, I^2 86%; p=0.008) for OS in 2 studies(Miller et al., 2017, Sinicrope et al., 2009); and one assessed DSS, finding a significant association with longer survival (HR 1.28, 95%CI: 0.12-0.66) (Miller et al., 2017). The funnel plot was wide and one reason for this is that some of the results tended towards poorer survival and some towards better survival. No comment can be made regarding publication bias since the number of studies was too few.

In colorectal cancer, twenty-two independent studies (6611 patients) assessed survival association with FoxP3(Suzuki et al., 2010, Katz et al., 2013, Zeestraten et al., 2013, Kim et al., 2015b, Lavotshkin et al., 2015, Mori et al., 2015, Chen et al., 2016, Väyrynen et al., 2016, Schweiger et al., 2016, Loddenkemper et al., 2006, Salama et al., 2009, Nosho et al., 2010, Lee et al., 2013, Xu et al., 2013, Chen and Chen, 2014, Vlad et al., 2015, Wang et al., 2015a, Berntsson et al., 2017, Sideras et al., 2018, Frey et al., 2010, Ling et al., 2014, Richards et al., 2014): of which thirteen (5603 patients) found it to be significant for survival(Lee et al., 2013, Kim et al., 2015b, Chen et al., 2016, Väyrynen et al., 2016, Salama et al., 2009, Nosho et al., 2010, Xu et al., 2013, Vlad et al., 2015, Wang et al., 2015a, Berntsson et al., 2017, Frey et al., 2010, Ling et al., 2014, Richards et al., 2014). Nineteen independent studies (5220 patients) assessed intratumoural FoxP3(Suzuki et al., 2010, Katz et al., 2013, Zeestraten et al., 2013, Kim et al., 2015b, Mori et al., 2015, Chen et al., 2016, Väyrynen et al., 2016, Schweiger et al., 2016, Loddenkemper et al., 2006, Salama et al., 2009, Lee et al., 2013, Xu et al., 2013, Vlad et al., 2015, Wang et al., 2015a, Berntsson et al., 2017, Sideras et al., 2018, Frey et al., 2010, Ling et al., 2014, Richards et al., 2014), of which eleven studies (4898 patients) were significantly associated with survival(Chen et al., 2016, Väyrynen et al., 2016, Salama et al., 2009, Lee et al., 2013, Xu et al., 2013, Vlad et al., 2015, Wang et

al., 2015a, Berntsson et al., 2017, Frey et al., 2010, Ling et al., 2014, Richards et al., 2014). One of these papers found both negative and positive influence on survival depending on whether FoxP3 T-cells were found in the intraepithelial compartment or combined intraepithelial and stromal compartments, respectively(Xu et al., 2013). There was only one other study with significant results that assessed more than one intratumoural compartment for FoxP3, finding that both IE and ST FoxP3 were significant for longer survival(Richards et al., 2014). Six independent studies (1597 patients) assessed FoxP3 at the invasive margin(Kim et al., 2015b, Väyrynen et al., 2016, Ling et al., 2014, Richards et al., 2014, Schweiger et al., 2016, Sideras et al., 2018), with a positive survival association in four studies comprising 1281 patients(Kim et al., 2015b, Väyrynen et al., 2016, Ling et al., 2014, Richards et al., 2014). Of the three studies with significant findings that assessed both intratumoural FoxP3 and at the invasive margin, all found the presence of FoxP3 to be associated with better survival, regardless of tumour compartment assessed(Väyrynen et al., 2016, Richards et al., 2014, Ling et al., 2014). Four studies performed a combined assessment of FoxP3 at the invasive margin and intratumoural(Lavotshkin et al., 2015, Ling et al., 2014, Nosho et al., 2010, Chen and Chen, 2014), of which 2 found a significant positive association with survival(Nosho et al., 2010, Ling et al., 2014). Eleven studies (3822 patients) used an electronic method of assessment(Kim et al., 2015b, Katz et al., 2013, Lavotshkin et al., 2015, Chen et al., 2016, Väyrynen et al., 2016, Salama et al., 2009, Nosho et al., 2010, Lee et al., 2013, Vlad et al., 2015, Berntsson et al., 2017, Sideras et al., 2018), of which eight (3336 patients) found a significant association of FoxP3 with longer survival(Kim et al., 2015b, Chen et al., 2016, Väyrynen et al., 2016, Salama et al., 2009, Nosho et al., 2010, Lee et al., 2013, Vlad et al., 2015, Berntsson et al., 2017). Eleven studies (2789 patients) used a manual method of assessment(Xu et al., 2013, Suzuki et al., 2010, Zeestraten et al., 2013, Mori et al., 2015, Schweiger et al., 2016, Loddenkemper et al., 2006, Chen and Chen, 2014, Wang et al., 2015a, Frey et al., 2010, Ling et al., 2014, Richards et

al., 2014), of which five (2267 patients) found a significant effect of FoxP3 on survival(Xu et al., 2013, Wang et al., 2015a, Frey et al., 2010, Ling et al., 2014, Richards et al., 2014), one of which was both positive and negative(Xu et al., 2013). Eight studies assessed the presence of MSI: of which one found FoxP3 to be independent of MSI(Ling et al., 2014); two found MSI not to be independently significant for survival(Zeestraten et al., 2013, Salama et al., 2009); one study assessed FoxP3 in MSI and MSS subgroups separately and found FoxP3 to be significant in the MSS subgroup(Frey et al., 2010); two studies did not include MSI in multivariate analysis(Kim et al., 2015b, Mori et al., 2015); and two studies found that FoxP3 was not independent of MSI for survival(Väyrynen et al., 2016, Berntsson et al., 2017). Fifteen independent studies (4040 patients) used arbitrary data thresholds(Suzuki et al., 2010, Zeestraten et al., 2013, Kim et al., 2015b, Mori et al., 2015, Schweiger et al., 2016, Loddenkemper et al., 2006, Salama et al., 2009, Nosho et al., 2010, Lee et al., 2013, Xu et al., 2013, Vlad et al., 2015, Wang et al., 2015a, Sideras et al., 2018, Ling et al., 2014, Richards et al., 2014), of which nine studies (3357 patients) found a significant association with survival(Kim et al., 2015b, Salama et al., 2009, Nosho et al., 2010, Lee et al., 2013, Xu et al., 2013, Vlad et al., 2015, Wang et al., 2015a, Ling et al., 2014, Richards et al., 2014). Five studies (2434 patients) used data-driven thresholds(Katz et al., 2013, Chen et al., 2016, Väyrynen et al., 2016, Berntsson et al., 2017, Frey et al., 2010), four of which (2246 patients) were associated with longer survival(Chen et al., 2016, Väyrynen et al., 2016, Berntsson et al., 2017, Frey et al., 2010). For two studies (137 patients), the data threshold method was unclear(Lavotshkin et al., 2015, Chen and Chen, 2014).

Those studies meeting inclusion criteria for meta-analysis of FoxP3 in colorectal cancer are given in Table 1.5 (Figure 1.10). There were five studies in colorectal cancer assessing IT FoxP3 giving combined fixed effects HRs of 0.52 (95% CI 0.36-0.77) for DFS in 2 studies(Chen et al., 2016, Väyrynen et al., 2016), (no significant heterogeneity); 0.72 (95%

CI 0.65-0.80) for OS in 4 studies(Chen et al., 2016, Nosho et al., 2010, Salama et al., 2009, Väyrynen et al., 2016), (substantial heterogeneity, I^2 77%; p=0.004); and 0.47 (95% CI 0.37-0.61) for DSS in 3 studies(Nosho et al., 2010, Richards et al., 2014, Väyrynen et al., 2016), (no significant heterogeneity). Funnel plot appeared to be skewed to the left indicating a bias against smaller studies with no significant difference in survival outcome. There were three studies in colorectal cancer assessing IM FoxP3. One study assessed DFS finding IM FoxP3 significant for longer DFS (HR 0.42, 95% CI 0.19-0.93) (Väyrynen et al., 2016), whilst for OS and DSS combined fixed effects HRs were 0.47 (95% CI 0.35-0.63) for OS in 2 studies(Nosho et al., 2010, Väyrynen et al., 2016), (no significant heterogeneity); and 0.57 (95% CI 0.46-0.70) for DSS in 3 studies(Nosho et al., 2010, Richards et al., 2014, Väyrynen et al., 2016), (substantial heterogeneity, I^2 63%; p=0.07). Funnel plot contained too few studies to comment on publication bias.

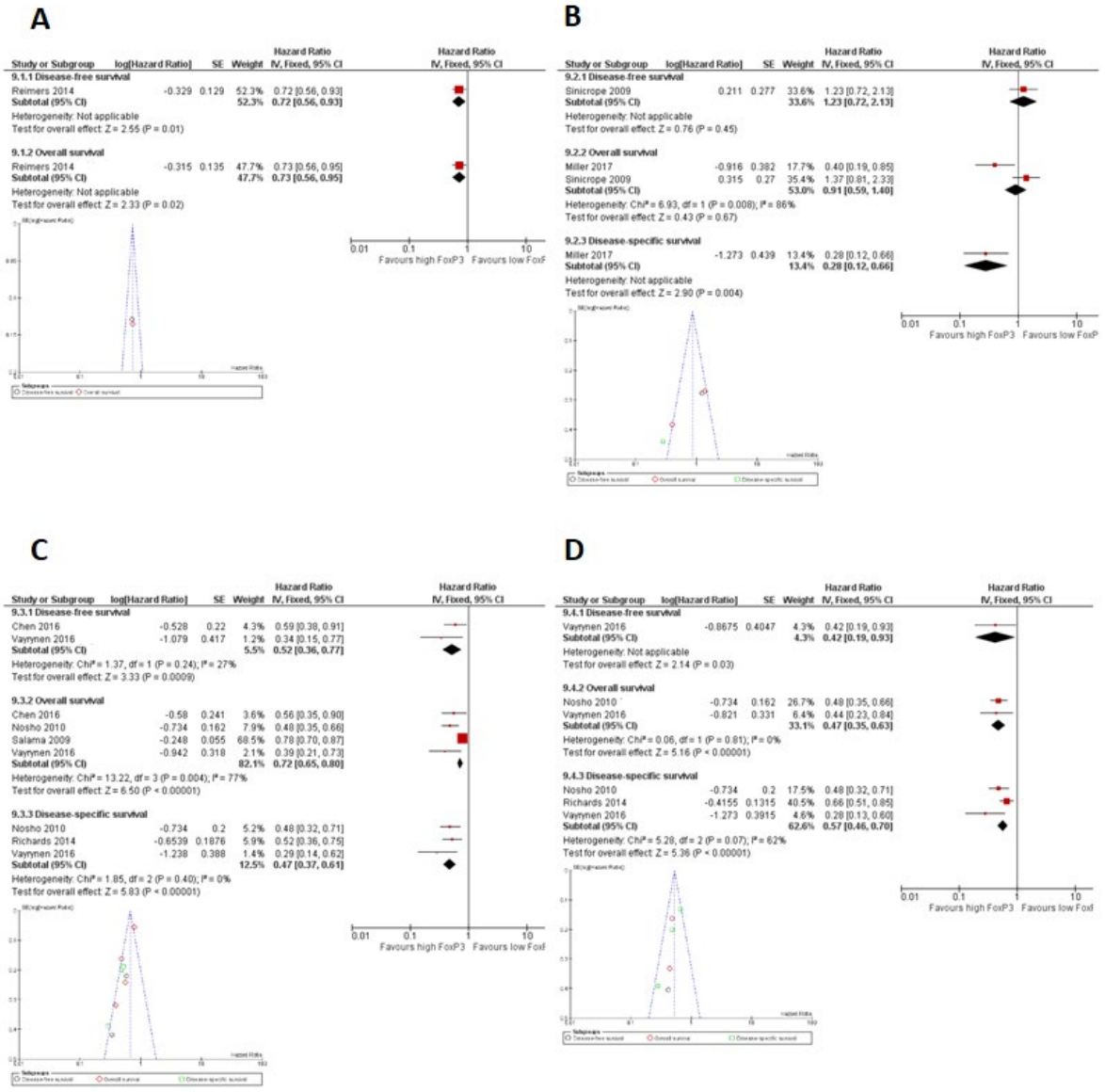


Figure 1.10. Forest plots and funnel plots for FoxP3 according to DFS, OS and DSS: A) IT in rectal cancer; B) IT in colon cancer; C) IT in colorectal cancer; D) IM in colorectal cancer

1.5.4.6 The "Immunoscore"

The Immunoscore® (HalioDx) is a patented score and a trademark of Inserm developed by Galon et al. over the last two decades(VERACYTE, 2019, Pagès et al., 2009, Pagès et al., 2018, Anitei et al., 2014b, Mlecnik et al., 2011, Galon et al., 2006). The score involves an electronic assessment of the presence of two T-cell markers in the intratumoural compartments or "tumour centre" (IE+ST) and at the IM giving 4 parameters, dichotomised in each region and for each cell type with a ROC curve into high or low. One point is assigned to each parameter scoring "high" and these points are added together to give a maximum score of 4 and a minimum of 0(Pagès et al., 2009). The original score used CD3 and CD45RO(Galon et al., 2006) as the two immune markers. Over time this evolved to use a combination of CD45RO and CD8(Pagès et al., 2009, Mlecnik et al., 2011), but was subsequently modified again to use CD3 and CD8(Anitei et al., 2014b, Pagès et al., 2018). This latter combination has been validated in a large international consortium with 2681 patients(Pagès et al., 2018). The score can be performed on either TMA(Pagès et al., 2009, Galon et al., 2006, Mlecnik et al., 2011, Anitei et al., 2014b) or whole sections(Pagès et al., 2018, Kirilovsky et al., 2016). This work has been replicated by other groups: some using the same software(Wirta et al., 2017), others using alternative electronic cell counting software(Markl et al., 2016, Wang et al., 2018, Yomoda et al., 2019, Nearchou et al., 2019), and others performing a manual assessment using the immunoscore method(Richards et al., 2014, Park et al., 2016a). Still others have adapted the score with additional items in the case of stage IV disease such as the addition of a Granzyme B marker (an additional marker of cytotoxic T-cell activation)(Halama et al., 2011), assessment of CD3 and CD8 in the tumour metastasis as well as the primary(Kwak et al., 2016, Liu et al., 2018) or addition of CD163 (an M2 macrophage marker)(Kwak et al., 2016), since stage IV disease is not effectively stratified by the immunoscore alone(Galon et al., 2006). In total, fourteen studies assessed a version of the Immunoscore in rectal, colon or colorectal cancer, although three studies had

overlapping populations(Anitei et al., 2014b, Pagès et al., 2009, Park et al., 2016a), leaving eleven independent studies comprising 4624 patients(Pagès et al., 2018, Markl et al., 2016, Mlecnik et al., 2011, Halama et al., 2011, Wirta et al., 2017, Wang et al., 2018, Yomoda et al., 2019, Kwak et al., 2016, Liu et al., 2018, Richards et al., 2014, Nearchou et al., 2019), of which only one (60 patients with stage IV disease) found no significant association with survival(Liu et al., 2018).

In rectal cancer, one study (83 patients) assessed the Immunoscore, which was significant for survival(Anitei et al., 2014b). They used an electronic method, counting CD3- and CD8-stained cells, and a data-driven threshold. MSI was not assessed and they used the original 5 group split(Anitei et al., 2014b). This study did not meet inclusion criteria for meta-analysis.

There were two studies (2827 patients) in colon cancer(Pagès et al., 2018, Markl et al., 2016), both of which were significant for longer survival. Both studies assessed primary colon cancer, although there were issues in one study with scoring CD8 in the tumour centre, leaving a total score of 3(Markl et al., 2016). Both studies used an electronic method of assessment, although one used the patented software(Pagès et al., 2018) and the other used freeware(Markl et al., 2016). Both studies assessed the presence of MSI: one found Immunoscore to be independent of MSI(Pagès et al., 2018); the other was unclear since they used a forward stepwise model for multivariate analysis(Markl et al., 2016). One study used an arbitrary threshold(Pagès et al., 2018), whilst the other used a data-driven threshold(Markl et al., 2016).

One study met inclusion criteria for meta-analysis (Table 1.4) for immunoscore in colon cancer and this was significant for DFS and OS (HRs 0.63, 95% CI 0.52-0.75 and 0.70, 95% CI 0.58-0.84, respectively)(Pagès et al., 2018).

In colorectal cancer, two groups used overlapping cohorts(Pagès et al., 2009, Park et al., 2016a), leaving nine independent studies comprising 1,797 patients(Mlecnik et al., 2011, Halama et al., 2011, Wirta et al., 2017, Wang et al., 2018, Yomoda et al., 2019, Kwak et al., 2016, Liu et al., 2018, Richards et al., 2014, Nearchou et al., 2019). Only one of these studies was not significant for survival and this was a small study of 60 patients with stage IV disease(Liu et al., 2018). There were four studies assessing the efficacy of the Immunoscore in metastatic disease, all of whom adapted the immunoscore to suit: two groups assessed the immunoscore only on liver metastases(Halama et al., 2011, Wang et al., 2018), one of whom added Granzyme B as a third marker in addition to CD3 and CD8(Halama et al., 2011); the other two assessed the immunoscore in the primary tumour centre, invasive margin and in the metastasis(Kwak et al., 2016, Liu et al., 2018); one of these also added in assessment of CD163 (as an M2 macrophage marker) in the primary in addition to the immunoscore(Kwak et al., 2016). Eight used an electronic method of assessment(Mlecnik et al., 2011, Halama et al., 2011, Wirta et al., 2017, Wang et al., 2018, Yomoda et al., 2019, Kwak et al., 2016, Liu et al., 2018, Nearchou et al., 2019), although two of these used freeware(Wang et al., 2018, Yomoda et al., 2019). One group used a manual method of assessment(Richards et al., 2014). Three groups assessed the presence of MSI: of which one found Immunoscore to be independent of MSI(Wirta et al., 2017); one found that MSI was not significant for survival(Park et al., 2016a); and one had only MSS tumours(Halama et al., 2011). Three studies employed an arbitrary data threshold method(Wang et al., 2018, Yomoda et al., 2019, Richards et al., 2014). Five studies employed data-driven thresholds, including the study that did not find a significant difference(Mlecnik et al., 2011, Wirta et al., 2017, Kwak et al., 2016, Liu et al., 2018, Nearchou et al., 2019). One study's data threshold method was unclear(Halama et al., 2011). Three groups used the originally proposed 5-category method of splitting cases(Mlecnik et al., 2011, Wirta et al., 2017, Richards et al., 2014); whereas the

remaining six dichotomised the data to give a high vs low score(Halama et al., 2011, Wang et al., 2018, Yomoda et al., 2019, Kwak et al., 2016, Liu et al., 2018, Nearchou et al., 2019).

Those studies meeting inclusion criteria for meta-analysis of Immunoscore in colorectal cancer are given in Table 1.5 (Figure 1.11). There were five studies in colorectal cancer assessing Immunoscore, giving combined fixed effects HRs of 0.49 (95% CI 0. 0.41-0.58) for DFS in 3 studies(Mlecnik et al., 2011, Pagès et al., 2009, Wirta et al., 2017), (substantial heterogeneity, I^2 91%; p<0.001); 0.61 (95% CI 0.53-0.70) for OS in 3 studies(Mlecnik et al., 2011, Pagès et al., 2009, Wirta et al., 2017), (substantial heterogeneity, I^2 89%; p<0.001); and 0.47 (95% CI 0.39-0.55) for DSS in 5 studies(Mlecnik et al., 2011, Pagès et al., 2009, Wirta et al., 2017, Park et al., 2016a, Nearchou et al., 2019), (substantial heterogeneity, I^2 86%; p<0.001). Funnel plot assessment was performed which showed that publications were significantly skewed to the left, with no studies showing no significant difference and the plot itself was wide indicating large variations in results. One study in particular was identified as an outlier(Pagès et al., 2009).

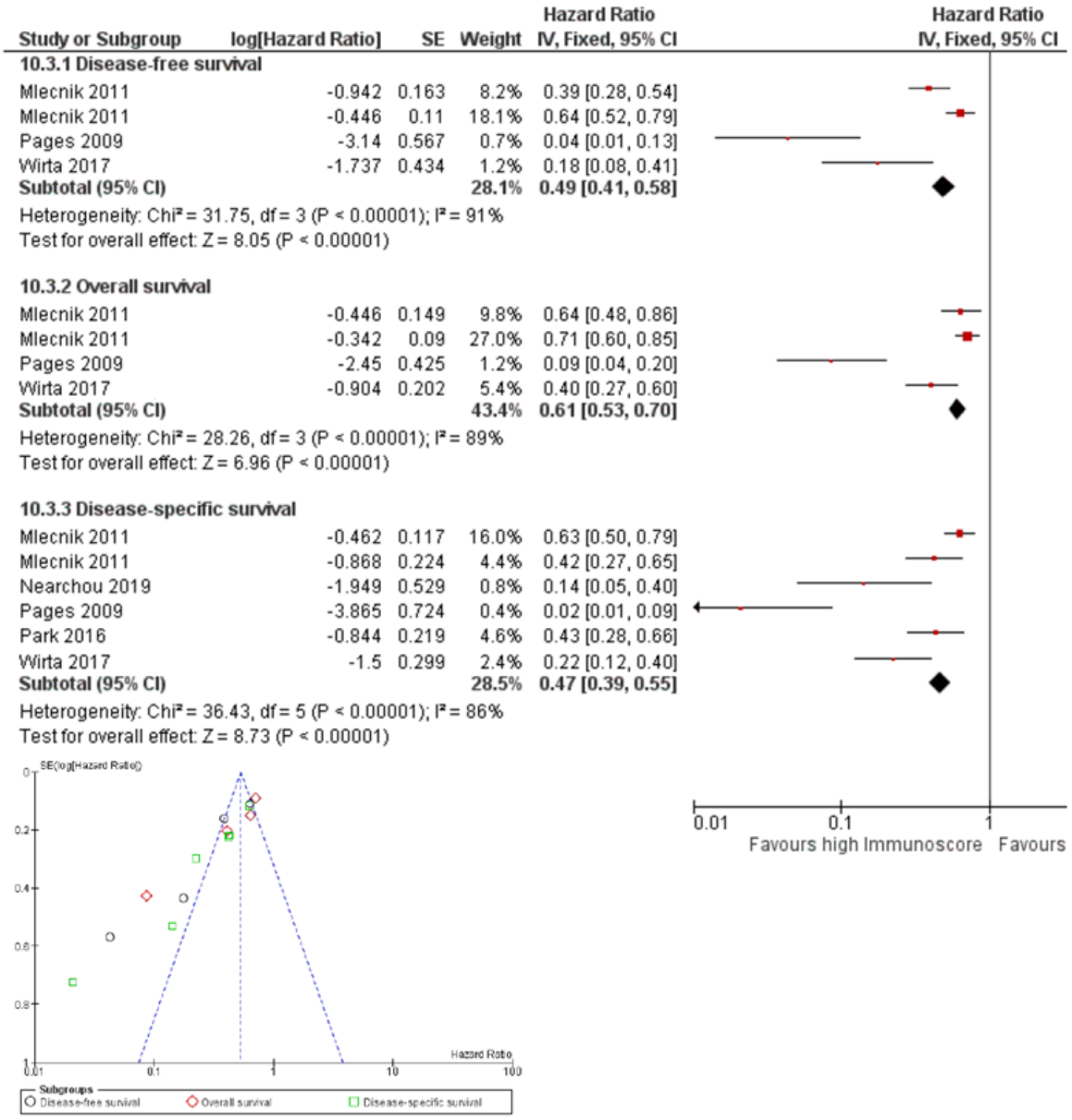


Figure 1.11. Forest plot and funnel plot for Immunoscore in colorectal cancer according to DFS, OS and DSS

1.5.4.7 Immunohistochemical Staining for CD20

CD20 (Cluster of differentiation 20) is a generic B-cell marker, whose role in anti-tumour pathophysiology is to interact with T-cells in co-ordinating the host defence by producing cytokines and chemokines in order to mount an immune response by recruiting/activating T-cells and acting as antigen presenting cells(Nelson, 2010, Tsou et al., 2016). Furthermore, mature B-cells produce antibodies directed against foreign entities(Nelson, 2010, Tsou et al., 2016). They tend to form tertiary lymphoid structures or lymphoid aggregates in colorectal cancer(Väyrynen et al., 2014), but are also found infiltrating the tumour itself(Baeten et al., 2006, Li et al., 2018a).

There were six independent studies (1491 patients) staining for CD20 in colorectal cancer(Bindea et al., 2013, Meshcheryakova et al., 2014, Chen et al., 2016, Baeten et al., 2006, Berntsson et al., 2016, Li et al., 2018a), of which five (1374 patients) found higher CD20 expression to be significant for longer survival(Bindea et al., 2013, Meshcheryakova et al., 2014, Chen et al., 2016, Berntsson et al., 2016, Li et al., 2018a). Four papers assessed CD20 in the intratumoural compartments(Chen et al., 2016, Baeten et al., 2006, Berntsson et al., 2016, Li et al., 2018a), of which three found a significant association with longer survival(Chen et al., 2016, Berntsson et al., 2016, Li et al., 2018a). Two papers assessed CD20 at the invasive margin(Meshcheryakova et al., 2014, Baeten et al., 2006), of which only one was significant for longer survival(Meshcheryakova et al., 2014). One study assessed CD20 on the whole slide (intratumoural as well as at the invasive margin) and this was significant for longer survival(Bindea et al., 2013). There were no studies with significant findings comparing more than one tumour compartment. Four studies used an electronic method of assessment, all of which were significant for survival(Bindea et al., 2013, Meshcheryakova et al., 2014, Chen et al., 2016, Li et al., 2018a). Two studies used a manual method of assessment(Baeten et al., 2006, Berntsson et al., 2016), of which one was significant for survival(Berntsson et al., 2016). Only one study assessed the presence of MSI

but neither included it in multivariate analysis(Berntsson et al., 2016). Two studies used an arbitrary data threshold(Baeten et al., 2006, Li et al., 2018a), of which one was significant for survival(Li et al., 2018a). Three used a data-driven threshold, all of which were significant for survival(Bindea et al., 2013, Chen et al., 2016, Berntsson et al., 2016). The data threshold method was unclear in one study(Meshcheryakova et al., 2014).

Those studies meeting inclusion criteria for meta-analysis of CD20 in colorectal cancer are given in Table 1.5 (Figure 1.12). There were three studies in colorectal cancer assessing IT CD20. One study assessed DFS finding IT CD20 significant for longer DFS (HR 0.62, 95% CI 0.40-0.96)(Chen et al., 2016), while two studies assessed OS giving a combined fixed effects HR of 0.66 (95% CI 0.52-0.89)(Berntsson et al., 2016, Chen et al., 2016), (no significant heterogeneity). Funnel plot provided no meaningful data regarding publication bias given small numbers.

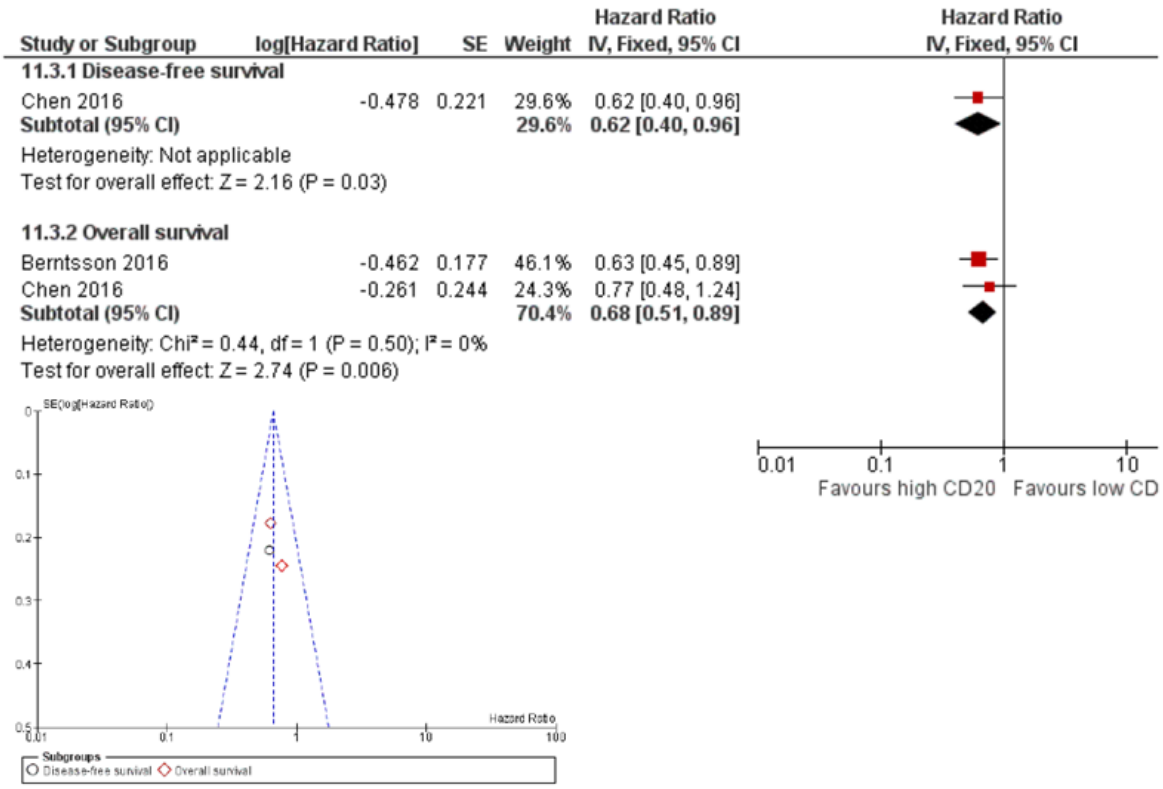


Figure 1.12. Forest plot and funnel plot for IT CD20 in colorectal cancer according to DFS and OS

1.5.4.8 Immunohistochemical Staining for Natural Killer Cell Markers

CD56 (Cluster of differentiation 56) and CD57 (Cluster of differentiation 57) are proteins commonly expressed on the surface of natural killer (NK) cells(Van Acker et al., 2017, Nielsen et al., 2013, Lopez-Verges et al., 2010), whose role as part of the innate immune system is to induce cell lysis although they may also have an immunoregulatory role(Vivier et al., 2008). CD56 is a more generic NK cell marker and can potentially also be expressed on other immune cells(Van Acker et al., 2017), whereas CD57 is a mature NK cell marker and is suggestive of terminal differentiation(Lopez-Verges et al., 2010).

One study (149 patients) assessed NK cells in rectal cancer, finding increased density to be associated with survival in the post-resection specimen(Alderdice et al., 2017). They measured intratumoural CD56 using an electronic method and an arbitrary data threshold. All the patients in this study were MSS. This study met inclusion criteria for meta-analysis (Table 1.6) and was significant for longer OS (HR 0.23, 95% CI 0.08-0.66)(Alderdice et al., 2017).

There were six studies (934 patients) in colorectal cancer(Coca et al., 1997, Chen et al., 2016, Nagtegaal et al., 2001, Menon et al., 2004, Tachibana et al., 2005, Liska et al., 2012), of which five (802 patients) were significant for longer survival(Coca et al., 1997, Chen et al., 2016, Menon et al., 2004, Tachibana et al., 2005, Liska et al., 2012). All six studies assessed the intratumoural compartments, of which four found intratumoural NK cells to be significant for survival(Coca et al., 1997, Chen et al., 2016, Tachibana et al., 2005, Liska et al., 2012). Two studies assessed NK cells at the invasive margin(Nagtegaal et al., 2001, Menon et al., 2004), one of which found a significant effect on survival(Menon et al., 2004). Only one paper with significant findings assessed NK cells in more than one tumour compartment, finding that their presence at the invasive margin was significant for survival, whereas their presence within the tumour was not(Menon et al., 2004). However, four other studies assessing intratumoural NK cells found their presence to be significant for longer

survival(Coca et al., 1997, Chen et al., 2016, Tachibana et al., 2005, Liska et al., 2012). All the studies in NK cells in colorectal cancer used a manual method of assessment apart from one, which used an electronic method(Chen et al., 2016). Only one study assessed the presence of MSI but did not include it in multivariate analysis(Menon et al., 2004). Two studies used an arbitrary data threshold, both of which were significant for survival(Coca et al., 1997, Menon et al., 2004). Two studies used a data-driven threshold, both of which were significant for survival(Tachibana et al., 2005, Chen et al., 2016). The threshold method was unclear for the remaining two studies(Nagtegaal et al., 2001, Liska et al., 2012). When broken down by antibody markers, neither of the papers assessing CD56 in colorectal cancer found any significant difference in survival(Nagtegaal et al., 2001, Menon et al., 2004), although the study in rectal cancer found the presence of CD56 positive cells to be associated with longer survival(Alderdice et al., 2017). All the studies assessing CD57 found a higher expression of CD57 to be significant for longer survival(Menon et al., 2004, Coca et al., 1997, Chen et al., 2016, Liska et al., 2012). The only paper comparing CD56 and CD57 staining in colorectal cancer found CD57 to be significant for longer survival, where CD56 was not(Menon et al., 2004). This suggests that CD57, which is believed to be suggestive of terminal differentiation(Lopez-Verges et al., 2010) is a more sensitive prognostic marker in colorectal cancer than CD56. One group stained for intratumoural Va24, a cell surface protein chain expressed on activated NK cells, which was also associated with longer survival(Tachibana et al., 2005).

Those studies meeting inclusion criteria for meta-analysis of CD57/Va24 in colorectal cancer are given in Table 1.5 (Figure 1.13). There were two studies in colorectal cancer assessing IT CD57/Va24, giving combined fixed effects HRs of 0.47 (95% CI 0.28-0.78) for DFS in 2 studies(Chen et al., 2016, Tachibana et al., 2005), (substantial heterogeneity, I^2 71%; p=0.06); and 0.48 (95% CI 0.28-0.84) for OS in 2 studies(Chen et al., 2016, Tachibana et al., 2005), (no significant heterogeneity). Funnel plot did not add meaningful data

regarding publication bias since numbers were too few. No studies met inclusion criteria for meta-analysis of IM CD56/57/Va24.

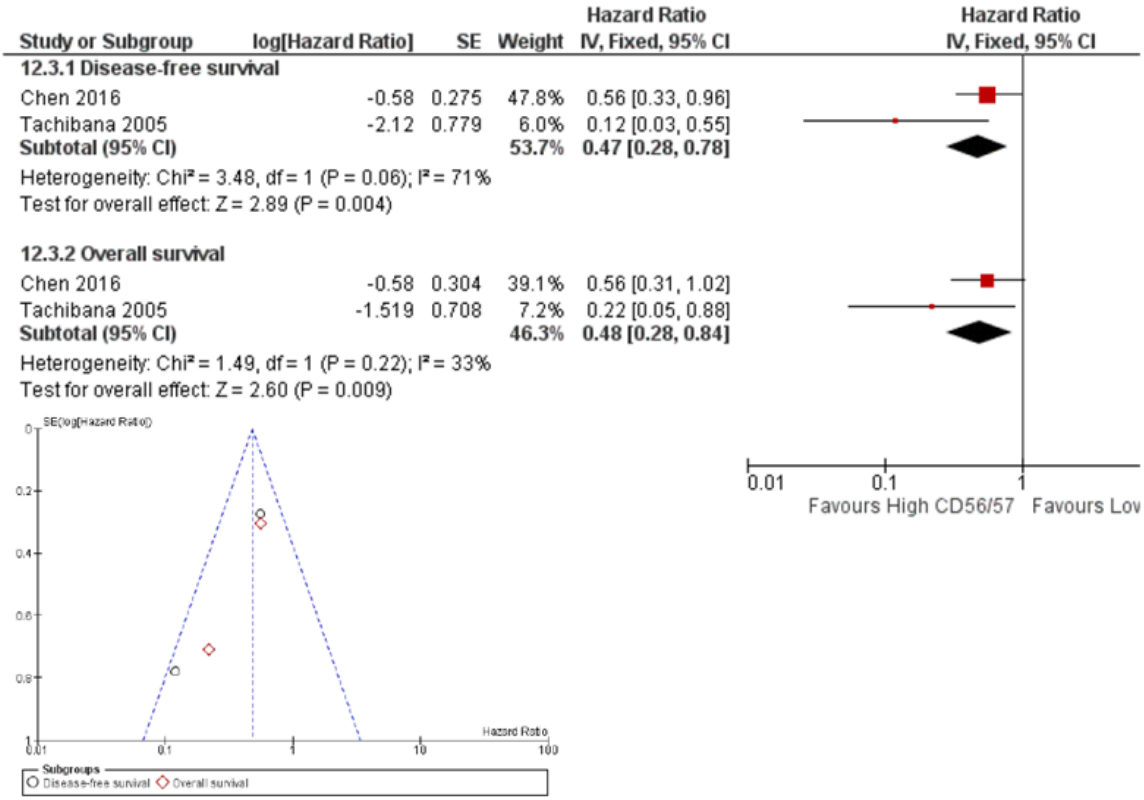


Figure 1.13. Forest plot and funnel plot for IT CD57/Va24 in colorectal cancer according to DFS and OS

1.5.4.9 Immunohistochemical Staining for Macrophage Populations

As part of the innate immune response, macrophages play a main role in phagocytosis and antigen presentation. They have been implicated in anti-tumour defence and T-cell recruitment(Pernot et al., 2014), but have also been implicated in a pro-tumour role(Shibutani et al., 2017a) and some have been shown to the express the immune checkpoint protein CD274 or Programmed death ligand 1(Lazarus et al., 2018), believed to play a role in tumour immune resistance(Gurjao et al., 2019). CD68 is a generic macrophage marker, whereas CD163 and CD206 are believed to be markers more specific to M2 tumour associated macrophages that have been linked with worse survival outcomes(Feng et al., 2019). There were twenty-two studies overall in colon or colorectal cancer although one study had an overlapping population(Edin et al., 2012), leaving twenty-one independent studies comprising 4879 patients(Feng et al., 2019, Herrera et al., 2013, Makkai-Popa et al., 2013, Väyrynen et al., 2016, Chen et al., 2016, Shibutani et al., 2017a, Li et al., 2018b, Li et al., 2018a, Kim et al., 2018, Lackner et al., 2004, Tan et al., 2005, Baeten et al., 2006, Nagorsen et al., 2007, Gulubova et al., 2013, Koelzer et al., 2016, Ding et al., 2018, Oberg et al., 2002, Funada et al., 2003, Forssell et al., 2007, Zlobec et al., 2011, Algars et al., 2012). Of these, eleven studies (2038 patients) found that higher infiltration of macrophages were associated with a better prognosis(Väyrynen et al., 2016, Chen et al., 2016, Lackner et al., 2004, Tan et al., 2005, Nagorsen et al., 2007, Gulubova et al., 2013, Koelzer et al., 2016, Oberg et al., 2002, Forssell et al., 2007, Zlobec et al., 2011, Algars et al., 2012), compared with five studies (1965 patients) finding a worse prognosis(Feng et al., 2019, Herrera et al., 2013, Shibutani et al., 2017a, Kim et al., 2018, Ding et al., 2018).

In colon cancer, two studies (1070 patients) assessed macrophage markers and survival. One study of 835 patients assessed CD68 and CD206(Feng et al., 2019). CD68 assessment in the intratumoural stromal compartment was not associated with survival, whereas CD206 was associated with worse survival(Feng et al., 2019). The other study assessed CD163 in the

intratumoural stromal compartment, which was associated with worse survival(Herrera et al., 2013). One used an electronic assessment method and a data-driven threshold(Feng et al., 2019), whereas the other used a manual method and an arbitrary threshold(Herrera et al., 2013). Neither paper assessed the invasive margin. Both assessed the presence of MSI: one found that MSI was not significant for survival(Feng et al., 2019); the other did not include MSI in survival analysis(Herrera et al., 2013). Neither study met criteria for inclusion in meta-analysis.

Sixteen independent studies (3528 patients) assessed CD68 in colorectal cancer: of which ten (1998 patients) were associated with an improved outcome(Väyrynen et al., 2016, Chen et al., 2016, Lackner et al., 2004, Tan et al., 2005, Koelzer et al., 2016, Gulubova et al., 2013, Oberg et al., 2002, Zlobec et al., 2011, Forssell et al., 2007, Algars et al., 2012), one (654 patients) was associated with a worse outcome(Kim et al., 2018) and five (876 patients) found no difference(Makkai-Popa et al., 2013, Li et al., 2018a, Baeten et al., 2006, Li et al., 2018b, Funada et al., 2003). The single study identifying an association of higher CD68 expression with worse survival assessed the expression in the IE and ST compartments separately, finding that the presence of CD68 in the IE compartment impacted negatively on survival, whereas CD68 in the ST compartment made no difference to survival(Kim et al., 2018). However, another three studies also measured CD68 in the IE compartment: of which two studies (187 patients) found no difference to survival(Lackner et al., 2004, Baeten et al., 2006); while one (201 patients) found it to be associated with improved survival(Koelzer et al., 2016). Twelve studies in total assessed intratumoural CD68(Makkai-Popa et al., 2013, Väyrynen et al., 2016, Chen et al., 2016, Li et al., 2018b, Li et al., 2018a, Kim et al., 2018, Lackner et al., 2004, Tan et al., 2005, Baeten et al., 2006, Gulubova et al., 2013, Koelzer et al., 2016, Oberg et al., 2002, Algars et al., 2012), of which five (796 patients) found it to be associated with better survival(Chen et al., 2016, Tan et al., 2005, Koelzer et al., 2016, Oberg et al., 2002, Algars et al., 2012), whereas one found it to be associated with worse survival,

as already mentioned(Kim et al., 2018). Eight studies assessed CD68 at the invasive margin: of which five found it to be associated with better survival(Väyrynen et al., 2016, Lackner et al., 2004, Gulubova et al., 2013, Forssell et al., 2007, Zlobec et al., 2011), compared with 3 that identified no survival association(Makkai-Popa et al., 2013, Baeten et al., 2006, Funada et al., 2003). Of the three studies that assessed CD68 both intratumoural and at the invasive margin, all found the invasive margin to be significantly associated with better survival, whereas intratumoural measurement was not significant(Väyrynen et al., 2016, Gulubova et al., 2013, Lackner et al., 2004). Five studies used an electronic method of assessment(Makkai-Popa et al., 2013, Väyrynen et al., 2016, Chen et al., 2016, Kim et al., 2018, Li et al., 2018a), of which two found CD68 to be associated with better survival(Väyrynen et al., 2016, Chen et al., 2016) and one with worse survival(Kim et al., 2018). Eleven studies used a manual assessment method(Li et al., 2018b, Lackner et al., 2004, Tan et al., 2005, Baeten et al., 2006, Gulubova et al., 2013, Koelzer et al., 2016, Oberg et al., 2002, Funada et al., 2003, Forssell et al., 2007, Zlobec et al., 2011, Algars et al., 2012), of which all were associated with improved survival apart from three(Li et al., 2018a, Baeten et al., 2006, Funada et al., 2003). Four studies assessed the presence of MSI: of which one found CD68 to be associated with better survival, independent of MSI(Zlobec et al., 2011); one found that CD68 was not independent of MSI(Väyrynen et al., 2016); while two did not include MSI in multivariate analysis(Kim et al., 2018, Koelzer et al., 2016). Eleven studies used an arbitrary data threshold(Makkai-Popa et al., 2013, Li et al., 2018b, Koelzer et al., 2016, Tan et al., 2005, Gulubova et al., 2013, Kim et al., 2018, Oberg et al., 2002, Funada et al., 2003, Forssell et al., 2007, Zlobec et al., 2011, Algars et al., 2012), of which only three found no impact on survival(Makkai-Popa et al., 2013, Li et al., 2018b, Funada et al., 2003). Two studies used a data-driven threshold, both of which found a significant association of CD68 with survival(Väyrynen et al., 2016, Chen et al., 2016). The data threshold method

was unclear for the other three studies(Lackner et al., 2004, Baeten et al., 2006, Li et al., 2018a).

Those studies meeting inclusion criteria for meta-analysis of CD68 in colorectal cancer are given in Table 1.5 (Figure 1.14). There were six studies in colorectal cancer assessing IT CD68, giving combined fixed effects HRs of 1.21 (95% CI 0.95-1.55) for DFS in 3 studies(Chen et al., 2016, Kim et al., 2018, Väyrynen et al., 2016), (substantial heterogeneity, I^2 81%; p=0.005); 0.91 (95% CI 0.75-1.11) for OS in 5 studies(Chen et al., 2016, Gulubova et al., 2013, Kim et al., 2018, Koelzer et al., 2016, Väyrynen et al., 2016), (substantial heterogeneity, I^2 77%; p=0.002); and 0.58 (95% CI 0. 0.38-0.89) for DSS in 2 studies(Väyrynen et al., 2016, Algars et al., 2012), (no significant heterogeneity). The funnel plot revealed most results to be grouped towards a positive influence of CD68 on survival, apart from a single large study indicating a negative effect on survival(Kim et al., 2018), which skews data to the right. There were three studies in colorectal cancer assessing IM CD68. One study assessed DFS and DSS finding IM CD68 significant for longer survival (HRs 0.43, 95% CI 0.19-0.96 and 0.40, 95% CI 0.20-0.81, respectively), (Väyrynen et al., 2016), whilst for OS the combined fixed effects HR was 0.48 (95% CI 0.36-0.64) for OS in 3 studies(Gulubova et al., 2013, Li et al., 2018a, Väyrynen et al., 2016), (moderate heterogeneity, I^2 48%; p=0.15). Funnel plot revealed no useful data given small numbers.

Six studies (1540 patients) assessed CD163 in colorectal cancer, of which two (508 patients) found it to be associated with an improved survival outcome(Nagorsen et al., 2007, Edin et al., 2012), two (241 patients) were associated with a worse outcome(Shibutani et al., 2017a, Ding et al., 2018), and two found no association with survival(Kim et al., 2018, Koelzer et al., 2016). Four studies assess intratumoural CD163(Kim et al., 2018, Nagorsen et al., 2007, Koelzer et al., 2016, Ding et al., 2018), of which one found it to be associated with better survival(Nagorsen et al., 2007) and one found it to be associated with worse survival(Ding et al., 2018), while the other two studies found no significant difference in survival outcomes.

Two studies assessed IM CD163, of which one was associated with worse survival(Shibutani et al., 2017a), while the other was associated with better survival(Edin et al., 2012). There were no studies that compared CD163 at the invasive margin versus intratumoural. Two studies used an electronic assessment method: one was associated with worse survival(Ding et al., 2018) and one was not associated with survival(Kim et al., 2018). Four studies used a manual assessment(Shibutani et al., 2017a, Nagorsen et al., 2007, Koelzer et al., 2016, Edin et al., 2012), of which two were associated with better survival(Nagorsen et al., 2007, Edin et al., 2012), while one was associated with worse survival(Shibutani et al., 2017a). Three studies assessed the presence of MSI, but none of them included this in survival analysis(Kim et al., 2018, Koelzer et al., 2016, Edin et al., 2012). All six studies used an arbitrary threshold apart from one that used a data driven threshold(Ding et al., 2018).

No studies on IT CD163/206 met inclusion criteria for meta-analysis. Two studies for IM CD163 were included in meta-analysis for colorectal cancer (Table 1.6, Figure 1.14): one assessed DFS finding a significantly worse survival outcome (HR 3.68, 95% CI 1.74-7.82), (Shibutani et al., 2017a); the other assessed DSS, with no significant survival difference (HR 0.66, 95% CI 0.42-1.05), but a trend towards better survival(Edin et al., 2012).

Two studies compared CD68 and CD163 stained cells(Kim et al., 2018, Koelzer et al., 2016). Both found CD68 to be significant for survival, where CD163 was not. However, one (654 patients) found CD68 was associated with worse survival(Kim et al., 2018), but the other one (201 patients) better(Koelzer et al., 2016). A further group studied the same population (468 patients) in two separate studies, one looking at CD68(Forssell et al., 2007) and the other at CD163(Edin et al., 2012), but they found that both cell subtypes were associated with better survival.

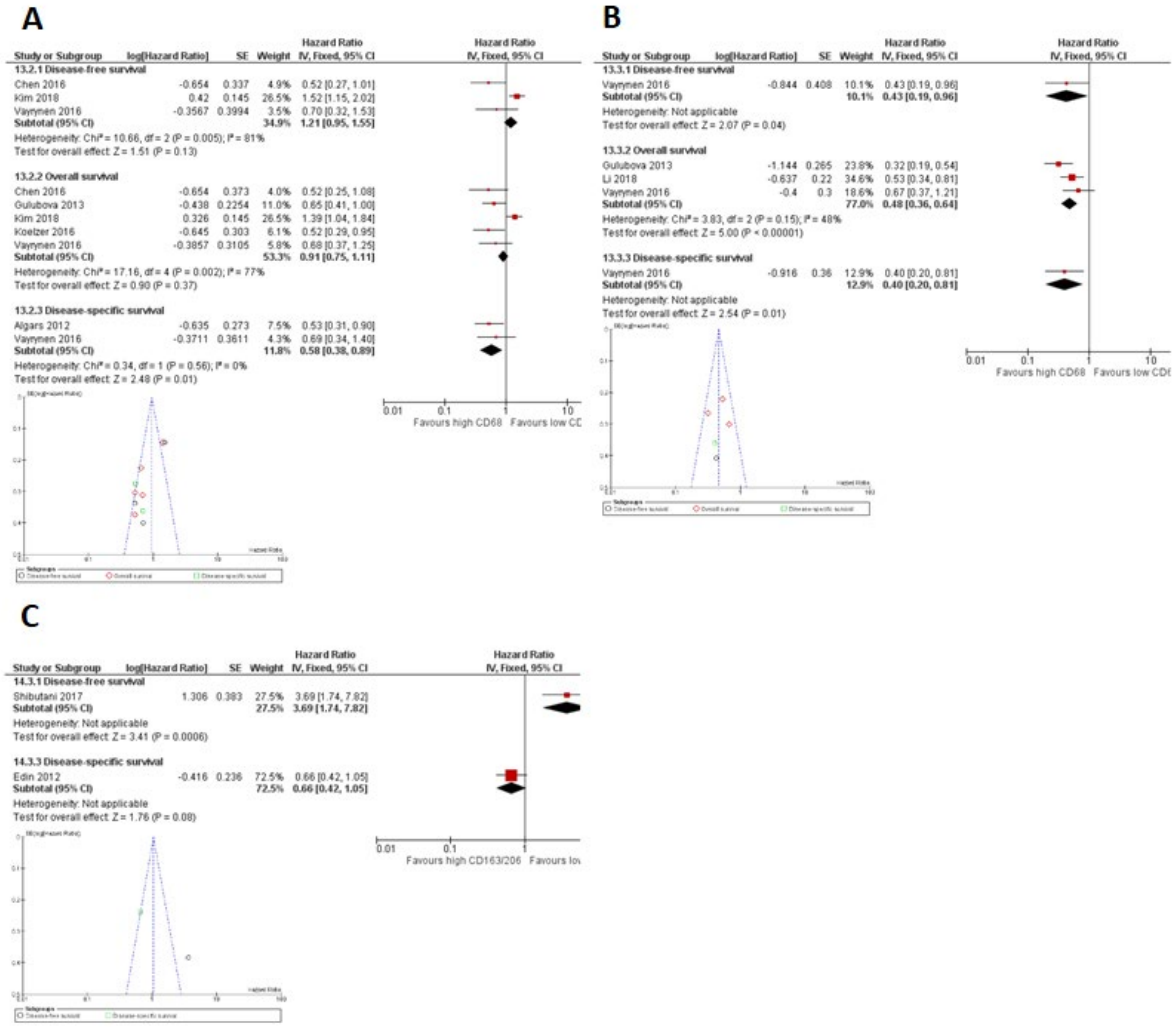


Figure 1.14. Forest plots and funnel plots for macrophage markers in colorectal cancer according to DFS, OS and DSS: A) IT CD68; B) IM CD68; C) IM CD163

1.5.5 Discussion and Conclusion

1.5.5.1 Discussion

The complex nature of the interaction between the tumour and host immune system has long been investigated. The findings from this meta-analysis show that, on the whole, a strong local inflammatory response is a positive prognostic indicator. Conversely, lack of a co-ordinated local inflammatory response results in a poor outcome.

The main aim of this review was to present evidence for survival outcomes based on assessment of the local inflammatory response in colorectal cancer. As a result, a secondary aim was to determine whether any particular inflammatory assessment had superior prognostic value, enabling a step towards incorporating assessment of the immune microenvironment in colorectal cancer into clinical practice.

In the past, some have advocated that not only the density of the local inflammatory response, but also the subtype of cells present and the location of these cells within the tumour microenvironment hold equal prognostic information(Galon et al., 2006, Pagès et al., 2008, Algars et al., 2012). However, in terms of cell type, the results of the meta-analysis presented show similar fixed effects summaries regardless of cell type assessed, with the exception of FoxP3 and macrophages. Furthermore, the fixed effects summaries are similar regardless of whether an H&E assessment method or IHC for a specific cell subtype is employed. For example, the fixed effects summary of KM for DSS in CRC was 0.40 (95% CI 0.29-0.55) compared with that of intratumoural CD3 (0.59, 95% CI 0.50-0.70) and CD8 (0.62, 95% CI 0.56-0.69), although there were twice the number the studies for CD3 and thrice the number of studies for CD8.

In terms of location of inflammatory cells, combined effects models were similar regardless of whether intratumoural assessment or assessment at the invasive margin were performed. In addition, those studies that compared IT assessment with IM did not agree on whether the

presence of inflammatory cells in one compartment conveyed superior survival advantage over the other. For example, of the 10 studies assessing CD8 in both intratumoural compartments (IT) and at the invasive margin (IM): 5 found both compartments to be significant for survival; 4 found higher IT CD8 significant, whereas IM was not; while 1 found higher IM CD8 significant, while IT was not.

When considering FoxP3 regulatory T-cells and macrophages, even these cell subtypes are associated with improved survival on the whole, but there does appear to be more heterogeneity in the literature regarding their positive or negative influence on survival outcomes. The negative impact of FoxP3 on survival has been attributed to the regulatory nature of this T-cell subset in dampening the effects of cytotoxic T-lymphocytes(Spence et al., 2015). In the case of macrophages, the negative prognostic effect has been attributed to the protumour effects of the "M2 macrophage" phenotype(Shibutani et al., 2017a, De Palma and Lewis, 2013). Indeed, Väyrynen et al have since published work suggesting that the ratio of M1:M2 macrophages is more important that the density of M2 macrophages or M1 macrophages, but that high density of M2 macrophages still associates with worse survival(Väyrynen et al., 2021). Both macrophages and cells expressing FoxP3 immune cell subsets may therefore be implicated in the immunoediting process that enables tumours to develop and evade or escape the host immunosurveillance(Dunn et al., 2004).

The fact that most, if not all of the inflammatory cells assessed contribute to an improved survival outcome indicates that it is the density of a healthy, functional and co-ordinated immune response of all cell types that drive an effective anti-tumour response(Nielsen et al., 1999, Richards et al., 2014). Conversely, it might be said that no cell type acting in isolation could effectively oppose tumour growth and metastasis. Hence those tumours with higher densities of infiltrating immune cells are associated with earlier stage, both in terms of depth of invasion and lymphatic and haematogenous spread(Richards et al., 2014, Pagès et al., 2008).

However, the question remains as to which method of assessing peritumoural inflammatory response is optimal in colorectal cancer. If concordance could be achieved regarding a universal assessment method, then an inflammatory assessment could be incorporated into the colorectal cancer dataset to compliment TNM staging and other clinicopathological variables and guide the most appropriate postoperative management for each patient. This issue is especially important given the increasing use of immunotherapies in identifying which patients would derive greatest benefit.

The most studied immune cell subtypes and therefore those with the greatest combined evidence behind their use are CD8, CD3 and FoxP3. FoxP3, given the heterogeneity in terms of survival outcomes is less ideal for a generalised marker of the anti-tumour immune response. Not only have the utilities of CD3 and CD8 individually been shown for colorectal cancer, both markers were associated with better prognosis when present in colon cancer and rectal cancer as well. Furthermore, the presence of CD3 and CD8 in preoperative biopsies was associated with better prognosis(Teng et al., 2015b, Ogura et al., 2018, Koelzer et al., 2014).

There are arguments, however, for the use of a simple H&E based method like the Klintrup-Mäkinen grade. Firstly, it is a validated method with similar fixed effects summaries to IHC methods. In agreement with this statement, Väyrynen et al. found that higher peri- and intra-tumoural densities of CD3, CD8, CD68, CD83, FoxP3 and neutrophils in CRC correlated with higher KM grade(Väyrynen et al., 2013). This finding was also shown in study validating the GMS, where higher CD3 and CD8 correlated strongly with strong KM(Alexander et al., 2020c). Secondly, KM is cost-effective, using routinely assessed slides in clinical pathology with no requirement for special stains. Therefore, in terms of global healthcare equity, the accessible nature of this method means that it would be available to all, regardless of a country's income status, across the globe(Beaglehole and Bonita, 2010). Having said this, there were fewer studies reporting this method and as a result the evidence

is less robust. There was also an element of interobserver variability reported in the published literature.

Similarly, CLR is able to stratify prognosis. However, with few studies investigating its prognostic role, coupled with the range of methods that have been used to assess CLR, in addition to the variability in thresholds used, the pooled evidence for CLR is weak. The same could also be said for TILs counted on H&E, CD4, CD45RO, CD20, and NK cell markers. All of these assessments had an overall positive survival effect, but the evidence was not strong enough to make any recommendations.

Furthermore, if an IHC method was preferred, the next question would be to agree a standardised assessment approach. There are those who have advocated for digital pathology and the use of patented software to achieve a standardised method with excellent reproducibility(Pagès et al., 2018). However, the rationale for this approach hinges on the fact that it matters which tumour compartment the inflammatory cells are found in, but the published literature displayed here does not support this theory. Furthermore, the incorporation of this method of assessment into routine clinical practice necessitates not only that all colorectal cancer cases are assessed in pathology laboratories with digital pathology facilities, but also that the patented software is purchased on a global scale. In addition, there is evidence of significant publication bias regarding the immunoscore, although the recent large prospective consortium trial provides high quality evidence that it does stratify survival(Pagès et al., 2018).

There are a range of methods of quantifying the extent of infiltration of CD3 and CD8, from semiquantitative scoring and manual counts to digital image analysis and automated counting. Around half of the studies included in meta-analysis for both CD3 and CD8 used a manual assessment and this did not impair their ability to identify those with a higher inflammatory infiltrate, which would suggest that an electronic assessment is not essential to the implementation of an IHC-based method of assessing host anti-tumour immune

response in routine clinical practice. The only study to directly compare manual and electronic assessment of IHC for CD3 found a good intraclass correlation co-efficient between manual and automated cell counting(Väyrynen et al., 2012).

MSI is a known confounding factor in colorectal cancer for any inflammatory assessment due to the greater inflammatory response that these tumours stimulate, a feature that has been attributed to greater quantities of neo-antigen generation(Wagner et al., 2018). Having said this, nearly half of the included studies did not assess for the presence of MSI and even less included this in multivariate analysis. However, in many studies MSI was not found to influence survival and in those where it did, there were several studies that showed that the survival benefit offered by a strong anti-tumour immune response was independent of MSI.

In the same way as patients with MSI and stage II disease are unlikely to require adjuvant therapy(Kawakami et al., 2015), those with a dense local inflammatory response would theoretically be offered similar protection.

1.5.5.2 Conclusion

Based on the weight of published evidence available, there was consensus from the published literature that the local inflammatory response was associated with longer survival. There did not appear to be any superiority of the use of IHC assessment of individual (or combined) inflammatory cells over the use of simple H&E-based local inflammatory cell scoring systems. It seemed reasonable, therefore, to take the Klintrup-Mäkinen grade forward in terms of its applicability to clinical practice.

1.6 ASSESSMENT OF THE MESENCHYMAL PHENOTYPE

The mesenchymal phenotype in colorectal cancer, which corresponds to the CMS 4 subtype, accounts for around 25% of all colorectal cancers(Guinney et al., 2015a). The predominant mutations in this subtype are APC (65%), TP53 (55%), and KRAS (40%) with a small proportion of BRAF (5%). These tumours are found most commonly in the distal colon and tend to be at a late stage at time of diagnosis(Testa et al., 2020). The transcriptomic phenotype of these tumours is dominated by angiogenic, inflammatory and immunosuppressive signatures(Becht et al., 2016). On histologic examination of these tumours, they are characterised by a high density of fibroblasts(Becht et al., 2016), otherwise described phenotypically as being high in tumour associated stroma(Testa et al., 2020). Still others have reported that tumour associated stroma itself bears a strong transcriptomic signature of epithelial-mesenchymal transition(McCorry et al., 2018).

Many groups have investigated the phenotypic element of tumour associated stroma, an extracellular matrix made up of mesenchymal cells, vascular endothelial cells, fibrous tissue, growth factors and chemokines(Werb and Lu, 2015).The presence of a greater percentage of tumour stroma has been shown to be a poor prognostic marker(Mesker et al., 2007, Huijbers et al., 2013, Park et al., 2014, Vogelaar et al., 2016).Whilst it has been made clear that higher tumour stromal percentage has a negative impact on outcomes in colorectal cancer, it remains unclear where tumour stroma arises from, whether these are local host fibroblasts that have been recruited by the tumour and transformed by a variety of chemokines, or whether these are in fact tumour cells that have undergone epithelial-mesenchymal transition (EMT) is unknown(Conti and Thomas, 2011). Mechanisms that explain the worse clinical outcomes for high stromal tumours have been proposed. These include a greater ability for neo-angiogenesis, thereby facilitating haematogenous metastases(Dvorak, 2015). In addition, tumour stroma may also play a role in facilitating tumour invasion and preventing apoptosis(Conti and Thomas, 2011, Werb and Lu, 2015, Becht et al., 2016). A further

mechanism for the poor prognosis in these tumours may be the higher levels of tumour budding that have been linked to high tumour stroma percentage(van Wyk et al., 2016), which is also believed to represent EMT(Grigore et al., 2016, Li et al., 2018a).

However, more recent research assessing the transcriptomic signature from tumour stroma has suggested that the high EMT signature of these tumours is largely related to the stroma and therefore may not represent tumour associated EMT at all(McCorry et al., 2018).

1.7 THE GLASGOW MICROENVIRONMENT SCORE (GMS)

The Glasgow Microenvironment Score(Park et al., 2015) was first described in a study published in 2015 by Park et al.. The score combined the immune phenotypic element of Klintrup-Mäkinen grade (KM) with the mesenchymal phenotypic element of Tumour Stroma Percentage (TSP). In a cohort of 307 stage I-III colorectal cancers, the scores were combined in the following way. Those patients with a strong KM were shown to have a better prognosis overall in terms of cancer-specific survival. Most of the patients with strong KM had low TSP. There was a very small group with strong KM and high TSP, but these did not have a statistically worse survival than the remaining patients with strong KM and therefore these two groups were combined (GMS 0). Those with weak KM were sub-divided according to TSP status, since weak KM with high TSP tumours were found to convey the worst prognosis (GMS 2), while those tumours with weak KM and low TSP were found to have intermediate cancer-specific survival outcomes (GMS 1).

Table 1.7. Summary of Glasgow Microenvironment Score categories

KM	TSP	Prognosis	GMS
Strong	Low	Good	GMS 0
Strong	High		
Weak	Low	Intermediate	GMS 1
Weak	High	Poor	GMS 2

Thus, phenotypically, the GMS may be considered to reflect the CMS subtypes with GMS 0 representing CMS 1, GMS 1 representing CMS 2 and 3 and GMS 2 representing CMS 4. Indeed, Roseweir et al.(Roseweir et al., 2020) used these features in the development of the "histologic phenotypic subtypes" with the addition of Ki67 immunohistochemical (IHC) staining as a marker of cell proliferation to divide the GMS 1 category into canonical (representative of CMS 2 with higher proliferation rate) or latent (representative of CMS 3, the metabolic category with lower proliferation rate). As expected, this histological

phenotypic subtype score, or modified GMS showed a similar stratification to that demonstrated by Park et al(Park et al., 2015). However, the addition of Ki67 to split GMS1 into high and low proliferation groups did not add any distinction in terms of survival outcomes(Roseweir et al., 2020).

1.8 SUMMARY AND AIMS

1.8.1 Summary

Colorectal cancer remains one of the most common cancers in the world and poses a significant disease burden worldwide with the 2nd highest cause of cancer-related mortality. There are now a greater number of therapeutic options available than ever before, particularly with the advent of immunotherapy for MSI cancers.

The genetic pathways leading to the development of colorectal cancers are becoming better understood, although there remain significant knowledge gaps, particularly related to the Serrated Pathway.

The consensus molecular subtypes have begun the process of allowing the ability to target certain therapies according to particular disease phenotypes although the debate is ongoing regarding the most efficacious means of assessing these phenotypes, not least the immune phenotype.

Global assessment of the immune infiltrate by Klintrup-Mäkinen grade had a similar, if not superior fixed effects summary on meta-analysis to assessment of CD3 or CD8 cell infiltrates, thereby justifying this simple and readily available assessment going forward.

Furthermore, the assessment of TSP is well recognised as a marker of a mesenchymal phenotype, although the relationship of TSP with markers of EMT is one that remains unclear. Some groups describe high EMT in CMS 4 patients and others report the high EMT transcriptomic signature as being related not to the tumour, but to the associated stroma(McCorry et al., 2018). Since the assessment of a mesenchymal phenotype (TSP) is an integral part of the GMS, the process of EMT might be key to understanding the mechanisms underlying the worse prognosis indicated by a mesenchymal phenotype (high TSP or GMS 2). Conversely, the better prognosis designated by a strong inflammatory response might be reflected in markers of EMT or absence thereof.

The Glasgow Microenvironment Score represents a composite histology-based phenotypic assessment of two well defined colorectal cancer phenotypes: the immune phenotype (CMS 1) and the mesenchymal phenotype (CMS 4). It has previously been shown to stratify survival in colorectal cancer into three distinct bands(Park et al., 2015).

Up to this point, the validity of the GMS had only been explored in a relatively small cohort of CRC from Glasgow Royal Infirmary(Park et al., 2015). It was possible, therefore, that the score would not produce the same results in independent patient cohorts. The score required to be tested in this context to see whether it retained its prognostic significance and independence of other clinicopathological features.

Furthermore, the pattern of colorectal cancer recurrence could also be related to the GMS phenotype and it could be that each GMS phenotype might give rise to a specific disease recurrence pattern or location. In particular, with the adverse features that are typically associated with GMS 2 (higher T- and N-stage and higher levels of venous invasion(Hansen et al., 2018)), the local recurrence rate was considered likely to be higher in this group.

The relevance of the GMS in terms of dictating adjuvant therapy had never been assessed and if a specific GMS category were able to guide adjuvant chemotherapy, then this would inform current and ongoing management of colorectal cancer. Therefore, the impact of GMS on different chemotherapeutic regimens required to be explored further.

With the rise of licenced immunotherapy agents, another important disease biomarker that has a role in predicting response to immunotherapy, used more in upper GI cancers, is the Programmed death ligand-1 (PD-L1 or CD274). However, the efficacy of this protein as a prognostic marker in CRC or indeed as an indicator of response to immunotherapy is unclear.

1.8.2 Hypotheses and Aims

To address the above areas of uncertainty regarding the relevance of the Glasgow Microenvironment Score, the following hypotheses were proposed:

1. Epithelial-mesenchymal transition markers were hypothesised to have different expressions according to the different phenotypic features assessed by the GMS
2. The GMS, as a histology-based assessment of the tumour microenvironment, remains relevant and able to stratify survival in independent cohorts
3. The GMS may give additional information regarding recurrence patterns and therefore aide decision-making regarding patient follow up
4. The phenotypic elements that are measured by the GMS may select patients who benefit from specific chemotherapeutic regimens

To examine these hypotheses, studies were performed in patients undergoing surgical resection of stage I-III colorectal cancer with curative intent. The aims of this research were as follows:

1. To assess the prognostic role of markers of EMT and the relationship between the GMS and markers of EMT in the context of colorectal cancer.
2. To assess the validity of GMS in independent patient cohorts to see whether it would retain its prognostic significance and independence of other clinicopathological features.
3. To examine the relationship between the GMS and patterns of recurrence.
4. To assess the associations of GMS with chemotherapy type and duration.
5. To investigate the impact of PD-L1 (CD274) as a prognostic biomarker in colorectal cancer and to assess its efficacy in the context of choice of immunotherapy

2. GENERIC METHODS

2.1 LITERATURE REVIEWS

Two separate systematic reviews and meta-analyses were performed. The first was titled: "The local inflammatory response in colorectal cancer – type, location or density? A systematic review and meta-analysis" and is published in Cancer Treatment Reviews(Alexander et al., 2020b). The review aimed to identify primary studies assessing the relationship between the host anti-tumour inflammatory response in CRC and survival. A search was made of PubMed, MEDLINE and EMBASE databases (limited to human studies, English language, between 1997 up to the present) and the titles and abstracts were reviewed to identify relevant full texts to be obtained, in addition to scrutiny of reference lists to identify any further studies.

The second systematic review and meta-analysis was titled "A meta-analysis of CD274 (PD-L1) assessment and prognosis in colorectal cancer and its role in predicting response to anti-PD-1 therapy" and is published in Critical Reviews in Oncology/Hematology(Alexander et al., 2021). This review aimed to identify all studies relating to survival in CRC and analysis of PD1 (programmed cell death protein-1) or PDL1 (programmed death ligand-1) expression. The initial search strategy and meta-analysis was the same as that above. However, in addition to this, all current trials registered with ClinicalTrials.gov for immunotherapies in CRC were identified and those with published results, whether in abstract form at conferences, or in published form in journals, were scrutinized with particular emphasis on those using PDL1 as a marker of disease response to immunotherapy in CRC.

For both meta-analyses, studies meeting REMARK criteria were included and relevant data were extracted for literature review(McShane et al., 2005). Those studies that also performed multivariate analysis and gave Hazard ratios for survival interaction were included in meta-analysis using REVMAN software, version 5.3. Confidence intervals crossing 1.0 were not considered significant and heterogeneity between studies was assessed with the I^2 value,

while a funnel plot was used to assess publication bias. An assessment of study bias was also performed according to REMARK criteria(McShane et al., 2005).

2.2 PATIENTS

2.2.1 Datasets upon which the contained research data are based

Several pre-constructed databases were utilised in the work undertaken in this thesis, both in study of the GMS and also assessment of inflammatory scores and EMT work:

- a combined database of two TMAs (referred to as the combined JP-AP TMA; *n*=1030),
    - one from patients who had surgical resections performed in Glasgow Royal Infirmary between 2000 and 2007 (the JP TMA, n=272);
    - the other from patients who had resections performed in other Glasgow hospitals (Stobhill, Gartnavel General and the Western Infirmary) between 2000 and 2007 (the AP TMA, n=758);
- the TransSCOT database, from patients who were enrolled in the SCOT arm of the IDEA 3-months vs 6-months adjuvant chemotherapy trial (*n*=2912);
- a prospectively collated, previously published(Park et al., 2016b), database of 1000 patients who had resections performed in Glasgow Royal Infirmary between 1997 and 2013 (referred to as the GRI-CRC-TMA; *n*=1000)

2.2.2 GRI-CRC-TMA construction and slide scanning

One thousand previously selected patients, derived from a previously published cohort of CRC resections in GRI between 1997 and 2013(Park et al., 2016b), were identified for construction of the GRI-CRC-TMA. In order to construct this TMA and also achieve the same H&E based scoring, the slides from old cases were pulled by laboratory staff. All slides

from full cases were reviewed and those from the deepest point of invasion were identified and scanned onto the electronic server for H&E-based whole section scoring. Furthermore, the slides also had tumour areas marked for construction of the new TMA and tumour blocks from these identified slides were looked out of the archive.

2.3 CLINICOPATHOLOGICAL DATA

Clinicopathological data were already collected by previous researchers in the unit from pathology reports and patient notes (both electronic and paper). However, I also played my part in the prospective maintenance of the database, entering all of the clinicopathological data for new patients having colorectal cancer resections, in order that future researchers might benefit from an accurate prospective database.

Survival data endpoints were: cancer-specific survival (CSS) defined as time from surgery to death from CRC-cause; overall survival (OS) defined as time from surgery to death from any cause; relapse-free survival (RFS) defined as time from surgery to recurrence of cancer or death from CRC-cause; and disease-free survival (DFS) defined as time from surgery to recurrence or death from any cause.

Mismatch repair (MMR) status for the combined JP-AP TMA was already known from previous researchers' work (Arfon Powell and James Park).

2.4 H&E-BASED SCORING

2.4.1 Klintrup-Mäkinen grade (KM)

Scoring of KM and TSP for the combined JP-AP TMA, as well as for the TransSCOT patients was already performed on whole sections (prior to TMA cores being taken) by Antonia Roseweir. I performed the KM and TSP scoring on the GRI-CRC-TMA patients on whole H&E sections where these were available (n=849), and a proportion of these were co-scored by Hester van Wyk. All scoring was performed on electronically scanned slides using

digital slide viewing software, initially Slidepath Digital Image Hub (Leica Biosystems, Milton Keynes, UK) and subsequently NDPServe (Hamamatsu). KM was scored as previously described(Klintrup et al., 2005) by assessing the inflammatory cell infiltrate at the invasive margin of the tumour, in addition to the cell density and any tumour cell nest destruction. A strong reaction (original score 2 or 3) was taken as a band or cup-like formation of inflammatory cells at the invasive margin with some evidence of tumour cell nest destruction (Figure 2.1), whereas a weak reaction (original score 0 or 1) was taken as a discontinuous inflammatory reaction or few cells with no evidence of cell nest destruction (Figure 2.2).

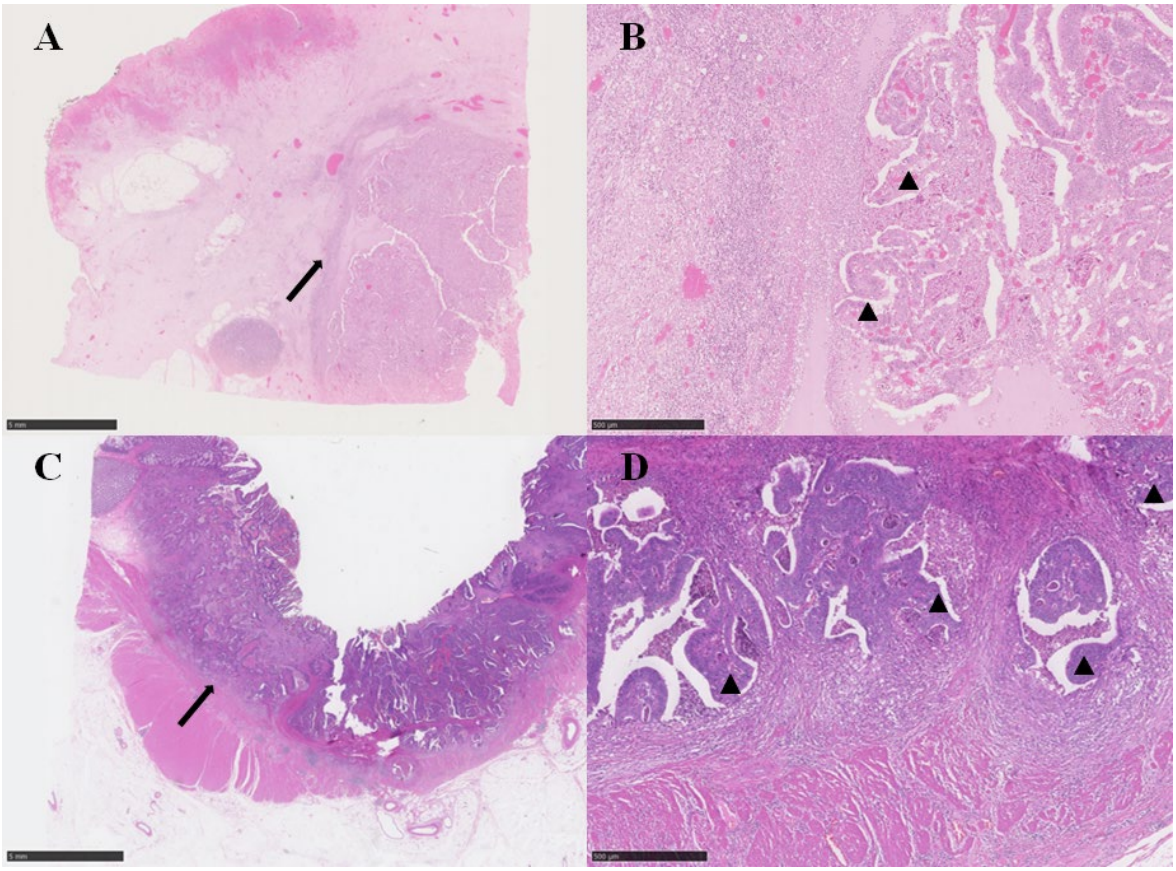


Figure 2.1. Two cases of strong KM with black arrows in A and C (at x10 magnification) demonstrating a continuous band of inflammatory cells and black triangles in B and D (at x100 magnification) demonstrating tumour nest destruction. Bars in A and C = 5mm. Bars in B and D = 500um

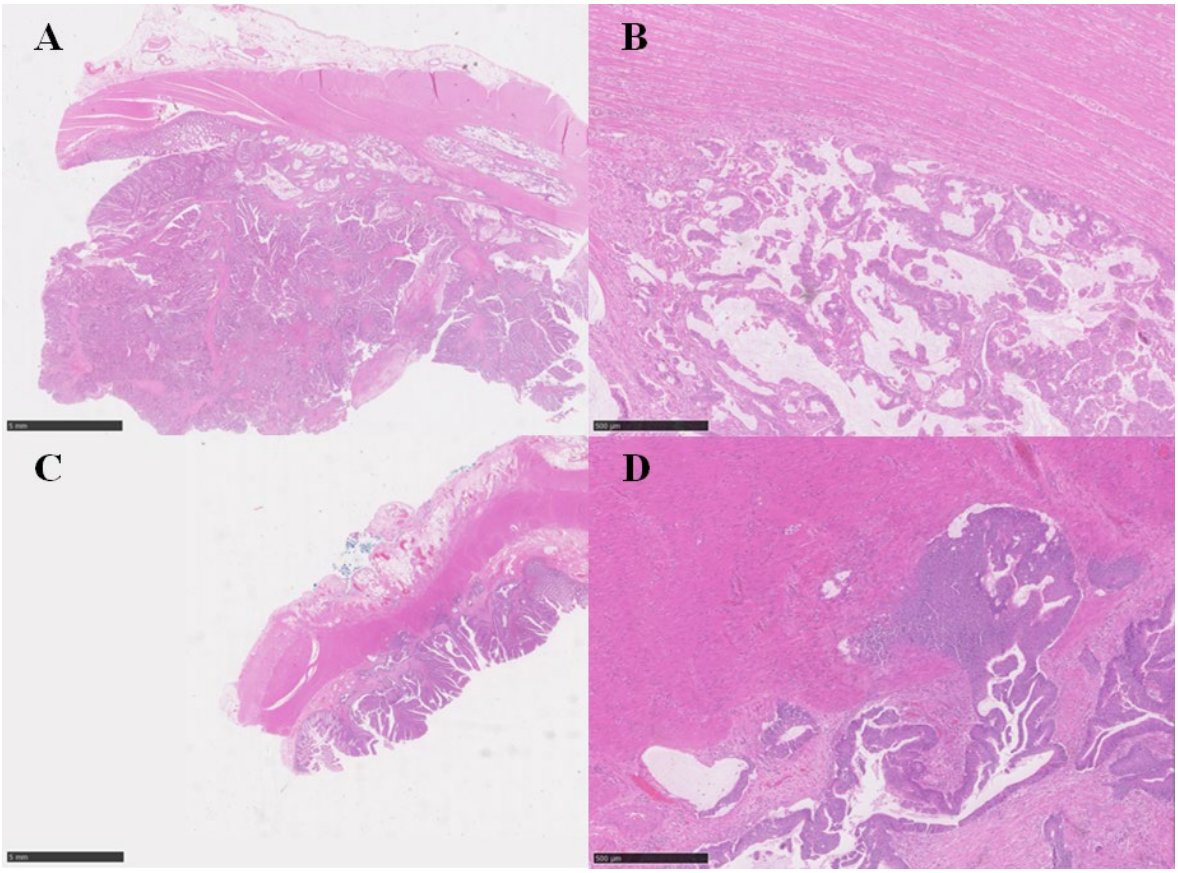


Figure 2.2. Two cases of weak KM with no evidence of inflammatory cells at invasive margin and no evidence of tumour next destruction. Bars in A and C = 5mm (x 10 magnification). Bars in B and D = 500um (x100 magnification)

2.4.2 Tumour stromal percentage

TSP was scored as previously described(Mesker et al., 2007), by assessing the percentage of tumour stroma to carcinoma percentage, ignoring areas of high mucin, at x100 magnification. Tumour stroma was scored in 10% increments. A score of more than 50% stroma was considered high stroma (Figure 2.3), whereas 50% or less was considered low stroma (Figure 2.4).

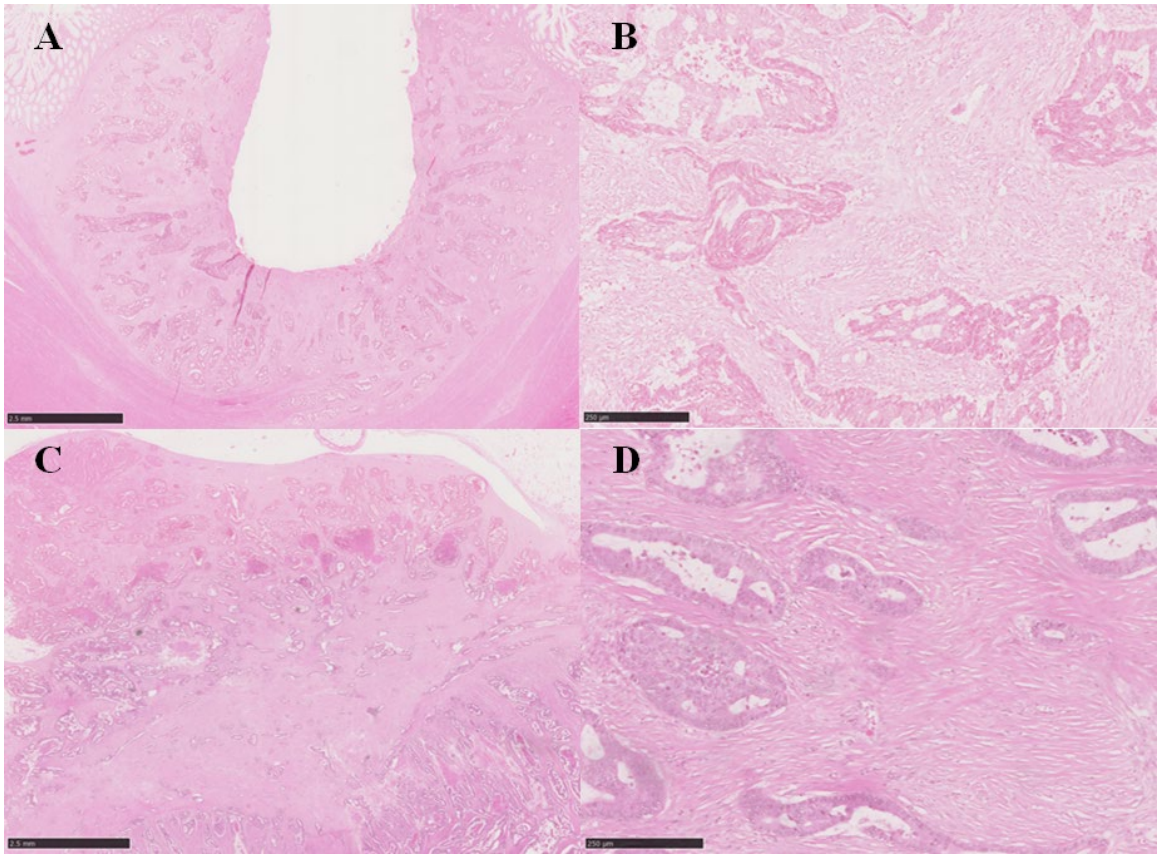


Figure 2.3. Two cases of high TSP at low magnification (x20) in A and C (Bars = 2.5mm) and high magnification (x200) in B and D (Bars = 250um) demonstrating greater than 50% stroma to tumour ratio.

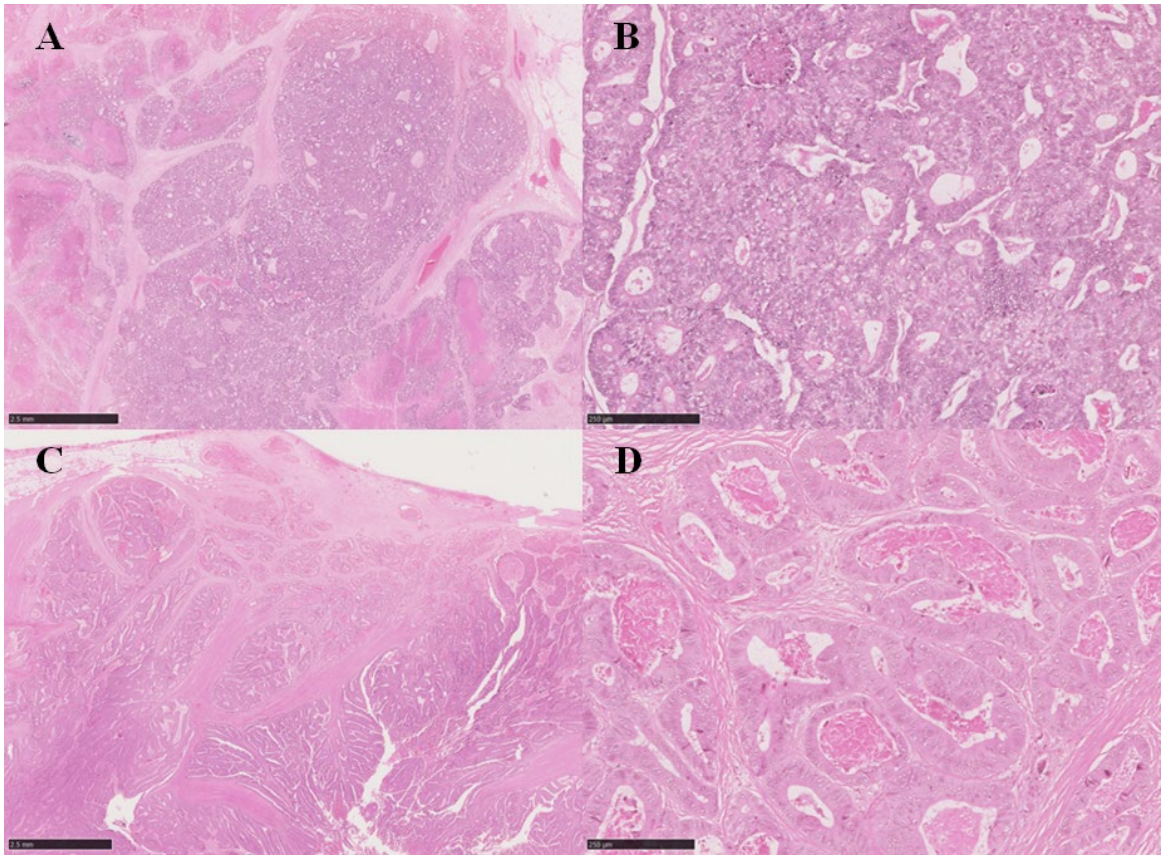


Figure 2.4. Two cases of low TSP at low magnification (x20) in A and C (Bars = 2.5mm) and high magnification (x200) in B and D (Bars = 250um) demonstrating less than 50% stroma to tumour ratio.

2.4.3 Crohn's-like reaction

Crohn's-like reaction (CLR) assessment was performed in three different ways as three different methods have been documented in the literature(Graham and Appelman, 1990, Ueno et al., 2013, Väyrynen et al., 2014). The number of lymphoid aggregates at the tumour's invasive margin were counted along with how many of these had germinal centres. Tumours with 3 or more aggregates and at least one germinal centre were considered "intense" and those with none were considered "absent". Those with intermediate numbers or no germinal centres were considered "mild" according to the Graham-Appelman (G-A) method(Graham and Appelman, 1990). The total number of aggregates was divided by the length in millimetres of the invasive tumour edge to give a density score, as per Väyrynen

et al.(Väyrynen et al., 2014). The largest aggregate was also measured in millimetres to give a size-based method, as per Ueno et al.(Ueno et al., 2013). Figure 2.5 demonstrates high CLR. For low CLR, please see Figure 2.2, which shows no evidence of lymphoid aggregates.

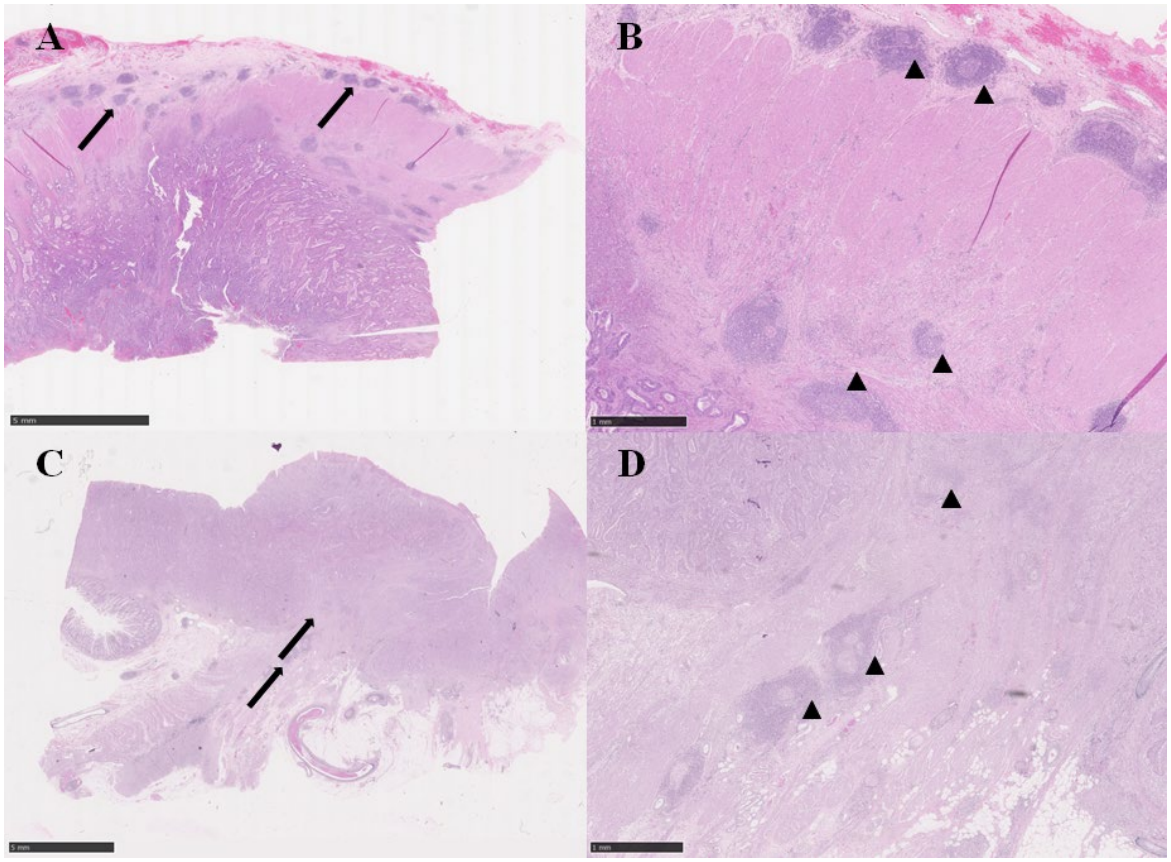


Figure 2.5. Two cases of high CLR with black arrows in A and C (at x10 magnification) demonstrating multiple lymphoid aggregates and black triangles in B and D (at x40 magnification) lymphoid aggregates with germinal centres. Bars in A and C = 5mm. Bars in B and D = 1mm

2.4.4 Tumour infiltrating lymphocytes (TILs)

Individual tumour infiltrating lymphocytes were also counted on H&E slides using an adaptation of a pre-defined method(Rozek et al., 2016, Richards et al., 2012, Ogino et al., 2009). Lymphocytes, dark purple ovals, positioned over cancer cell nests were counted manually in 10 high-power fields (HPF; 0.04mm^2 each) giving a total area assessed of 0.4mm^2. An average number of lymphocytes per HPF was then calculated. Figure 2.6 demonstrates high TILs, while Figure 2.7 displays examples of low TILs.

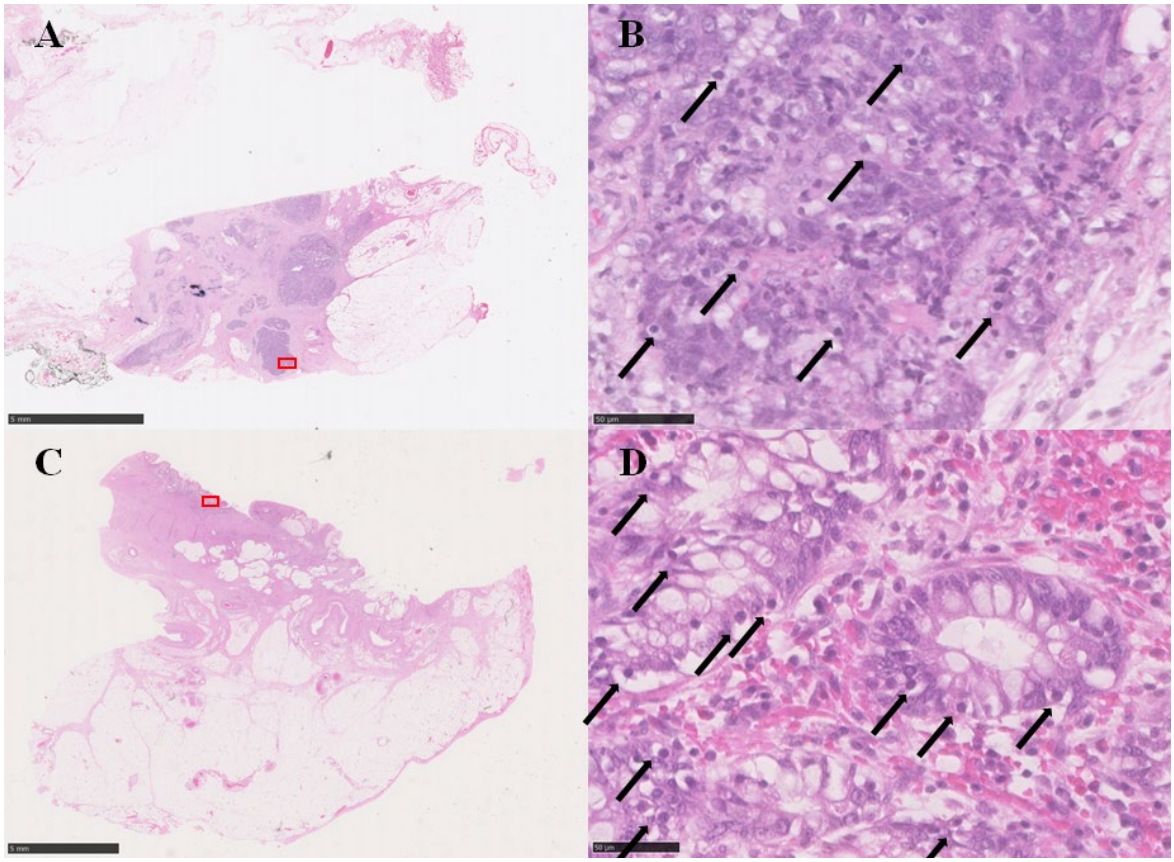


Figure 2.6. Two cases of high TILs in A and C (at x10 magnification). Red boxes indicate areas of magnification. Black arrows in B and D indicate intraepithelial lymphocytes (at x800 magnification). Bars in A and C = 5mm. Bars in B and D = 50um

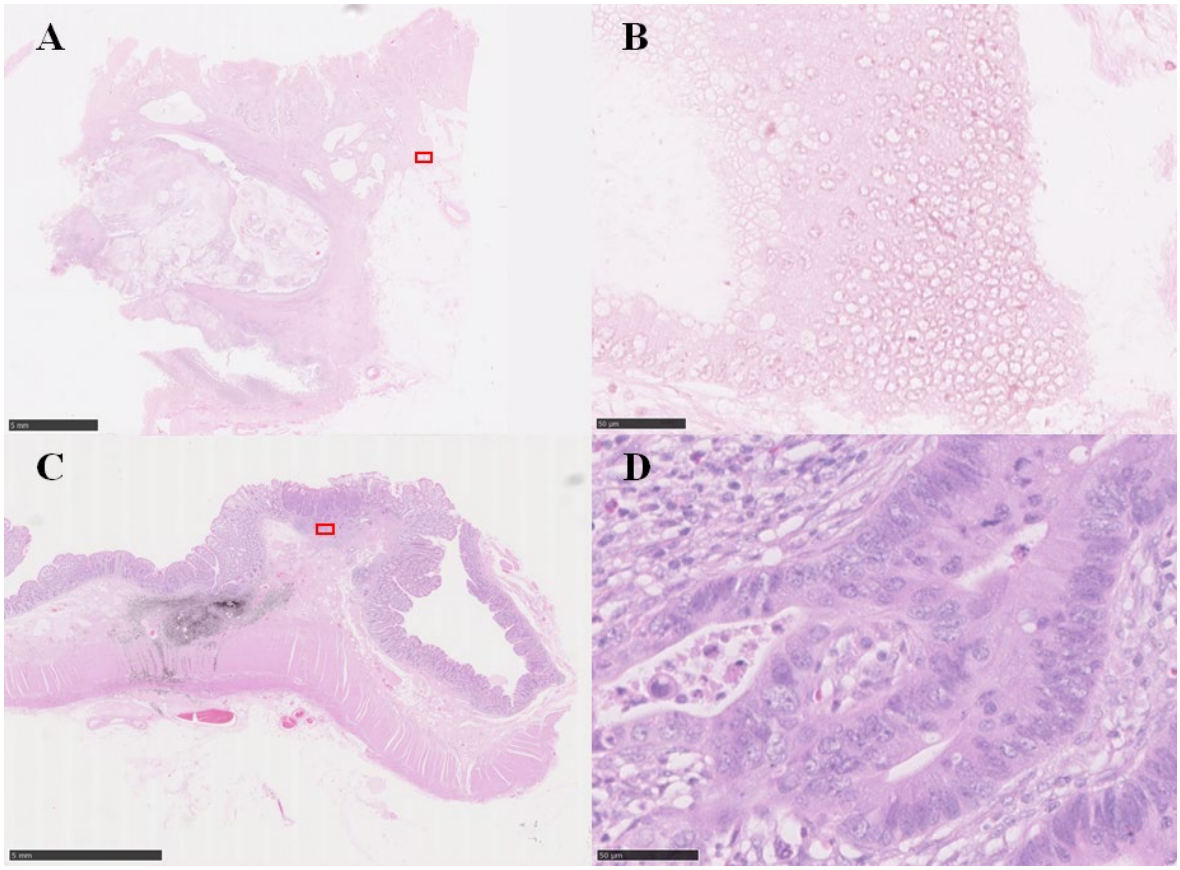


Figure 2.7. Two cases of low TILs in A and C (at x10 magnification). Red boxes indicate areas of magnification. There are no intraepithelial lymphocytes in B and D (at x800 magnification). Bars in A and C = 5mm. Bars in B and D = 50um

2.5 IMMUNOHISTOCHEMISTRY METHOD FOR EMT MARKERS

The immunohistochemical staining of the TMAs used for scoring EMT was performed by Niphat Jirapongwattana, supervised by Jean Quinn. My role was in scoring these TMAs after they had already been stained and scanned onto the shared server, accessed using NDPServe (Hamamatsu). IHC for the following markers of EMT was performed on the AP TMA: E-cadherin, B-catenin, Fascin, Snail and Zeb-1. Herein follows a brief description of the staining method.

TMAs were de-waxed using Histoclear and subsequently rehydrated using alcohol in decreasing concentrations. Antigens were subsequently retrieved as follows. In the case of E-Cadherin, Fascin, Snail and Zeb-1, a pressurised citrate buffer at pH 6.0 was used for 5

mins. 3% hydrogen peroxide was used for 20 minutes to block endogenous peroxidase activity. The TMAs were then incubated with 10% casein (Vector Laboratories) for 2 hours (Zeb-1, Fascin and Snail) or 30 mins (E-cadherin). Primary E-cadherin (1:500; BD Biosciences, 610182) and Zeb-1 (1:800, Sigma-Aldrich, HPA027524) antibodies were added and refrigerated at 4˚C overnight. For Fascin (1:100; Atlas Antibodies, HPA005723), and Snail (1:50; Abcam, ab53519) antibodies were added and kept for 2 hours at room temperature. Thereafter, TMAs were incubated with envision (DAKO) for half an hour in the case of E-cadherin, Fascin and Zeb-1; or with ImmPRESS anti-goat IgG for half an hour (Snail).

In the case of B-catenin, a pressurised water bath at pH 8.0 at 96˚C was used for 50 mins. 0.5% hydrogen peroxide was used for half an hour to block endogenous peroxidase activity. Following this, the TMAs were incubated for 30mins in 1% BSA. Primary B-catenin antibody (1:50; BD Biosciences, 610154) was added at room temperature for 2 hours. TMAs were then incubated for 2 hours with envision (DAKO). 3,3'-diaminobenzidine (DAB; Vector Laboratories) was used to achieve antibody visualisation until colour developed. Haematoxylin counter-staining was also used for all slides, which were then dehydrated using Histoclear and alcohol prior to mounting with DPX.

2.6 WEIGHTED HISTOSCORE (EMT MARKERS)

EMT-markers were scored using a weighted histoscore on the AP TMA. The weighted histoscore is a well-established method used to quantify expression of the protein of interest. Following immunohistochemical staining, expression within each cellular compartment (membrane, cytoplasm and nucleus) is scored separately by manual assessment of the proportion of the compartment stained at each density of staining (strong, moderate, weak or negative). These proportions are then multiplied as follows: (%tumour tissue with absent staining per core)x0 + (%tumour tissue with mild staining per core)x1 + (%tumour tissue with moderate staining per core)x2 + (%tumour tissue with strong staining per core)x3. This

method gave a range of scores per tissue core from 0 to 300. These scores were averaged over up to 4 cores per tumour. Examples of low and high scores for each marker are shown in Figure 2.6.

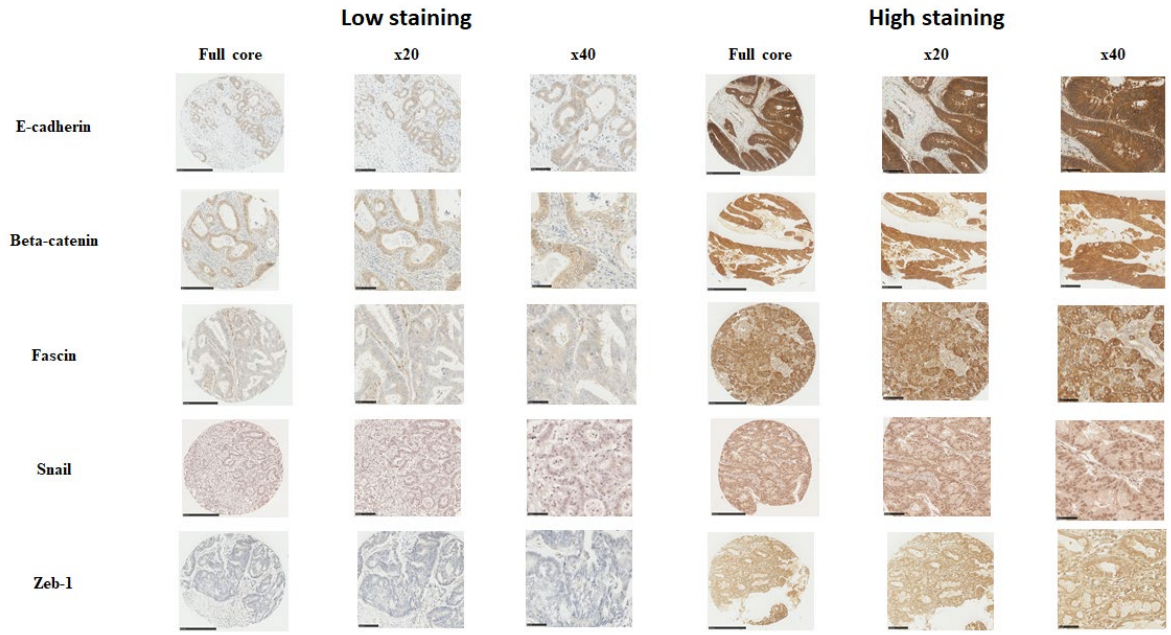


Figure 2.8. Representative images of low and high IHC staining for markers of EMT: E-cadherin, Beta-catenin, Fascin, Snail and Zeb1 in full TMA core (Bar = 250um), x20 magnification (Bar = 100um) and x40 magnification (Bar = 50um).

2.7 STATISTICAL ANALYSIS

SPSS versions 25.0, 27.0, and 28.0 (IBM, SPSS) were used for data analysis. Kaplan-Meier curves and log-rank analyses were used to compare CSS, OS, RFS and DFS. Hazard Ratios (HR) and 95% Confidence Intervals (CIs) were assessed using univariate Cox-regression analysis. For multivariate analysis, a backward conditional stepwise elimination model was used. A significance threshold of $p \leq 0.1$ on univariate survival analysis was used in identifying variables for multivariate analysis. Associations between categorical variables were evaluated using Pearson's chi-squared test. A p-value of less than 0.05 was considered statistically significant.

3. GMS AND MARKERS OF EPITHELIAL-MESENCHYMAL TRANSITION (EMT) IN THE CONTEXT OF COLORECTAL CANCER

Previously, the prognostic significance of EMT in CRC had been explored(Roseweir et al., 2019). However, the relationship of TSP with markers of EMT is one that remains unclear. Some groups describe high EMT in CMS 4 patients, while others have reported that the high EMT transcriptomic signature is related not to the tumour, but to the associated stroma(McCorry et al., 2018). Since the assessment of a mesenchymal phenotype (TSP) is an integral part of the GMS, the process of EMT might be key to understanding the mechanisms underlying the worse prognosis indicated by a mesenchymal phenotype (high TSP or GMS 2). Conversely, the better prognosis designated by a strong inflammatory response might be reflected in markers of EMT or absence thereof.

The phenomenon of EMT in its truest form is an embryological process essential for organogenesis(Micalizzi et al., 2010), whereas in the development of epithelial cancer metastases, it is thought to represent a process in which epithelial cells become less well-differentiated, losing cell-cell adhesion molecules (e.g. cadherins) and becoming more motile(Thiery, 2003, Micalizzi et al., 2010). It is believed that the cancer EMT process (henceforth referred to as EMT) gives rise to circulating tumour cells(Barriere et al., 2014). The cells that survive in the bloodstream and go on to form metastases in distant organs will be pluripotent cancer stem cells, enabling them to establish new tumours in distant sites(Reya et al., 2001).

There are a number of validated markers of EMT. E-cadherin is a cell surface protein functioning closely with the actin cytoskeleton that is involved in cell-cell adhesion, the loss of which is a marker of de-differentiation(Peinado et al., 2004). B-catenin, a member of the catenin family, which link cadherins to the actin cytoskeleton, is also a transcription factor and may be released when not linking E-cadherin to the cell membrane, although the process that drives B-catenin from the cell cytoplasm to the nucleus is

unclear(Brabletz et al., 2005). B-catenin is one of the proteins in the Wnt pathway and in embryological development is involved in both EMT and stem cell formation(Brabletz et al., 2005). The presence of nuclear B-catenin also reduces transcription of E-cadherin(Brabletz et al., 2005). Both higher nuclear B-catenin and lower membrane E-cadherin have been observed in tumour budding, a mesenchymal phenotype believed to be associated with EMT(Zlobec and Lugli, 2010). Both Snail and Zinc finger-E-box binding homeobox 1 (Zeb-1) are transcriptional factors that promote a mesenchymal phenotype and reduce the expression of membrane E-cadherin(Grigore et al., 2016). Fascin is a downstream target of B-catenin and is usually responsible for bundling of actin cytoskeleton but is upregulated in epithelial cancers(Machesky and Li, 2010) and results in increased cell motility and migration(Vignjevic et al., 2007).

Since the ability to identify the process of EMT in colorectal cancer will indicate which tumours may metastasise, a simple yet robust means of identifying such tumours is essential. In a previous study, our research group showed that a combination of these five markers was associated with survival in a cohort of colorectal cancer patients(Roseweir et al., 2019).

The aims of this section were to assess the prognostic role of markers of EMT in an independent cohort and the relationship between the GMS and markers of EMT. In particular, it was hypothesised that tumours with high immune infiltrates (GMS 0) would have lower expression of EMT markers, whereas those with a mesenchymal phenotype (GMS 2) may have a higher expression of EMT markers(Park et al., 2015, Alexander et al., 2020c).

3.1 SPECIFIC METHODS

3.1.1 Patient cohort

The AP TMA was used to explore the prognostic role of the five markers of EMT in CRC and also to assess their relationship with the GMS. Two-hundred and thirty-eight TNM II-III CRC specimens were identified retrospectively from this database All patients had undergone surgery with curative intent between 2000 and 2007. Those who had endoscopic or palliative procedures and those with involved surgical margins (R1) were excluded, as were those who died within 30 days of surgery and those who received neoadjuvant chemoradiotherapy. The primary endpoint was cancer-specific survival (CSS), defined as time from surgery to death from colorectal cancer. Survival data were available until the 1st July 2020.

3.1.2 Clinicopathological characteristics

Tumour budding(van Wyk et al., 2019) and MMR status(Powell, 2016) were already available for this cohort. The Petersen index was used to assess clinical risk as in clinical practice indicating low or high-risk stage II colorectal cancer(Petersen et al., 2002): venous invasion and peritoneal involvement were assigned a score of 1, while tumour perforation was assigned a score of 2. TNM II disease with Petersen index of 2 or higher, or TNM III disease was considered high-risk. Peritumoural inflammatory scores (KM grade) and tumour stromal percentage scores (TSP) were already available(van Wyk et al., 2019). These were combined as the GMS as previously described(Alexander et al., 2020c). In brief, strong KM and any TSP scored GMS 0; weak KM with low TSP scored GMS 1 and weak KM with high TSP scored GMS 2. The modified Glasgow Prognostic score (mGPS) was calculated using serum CRP (C-reactive protein) and albumin levels obtained in the 30 days before surgery or at the time of admission as previously described(Park et al., 2016b).

3.1.3 Immunohistochemistry and scoring

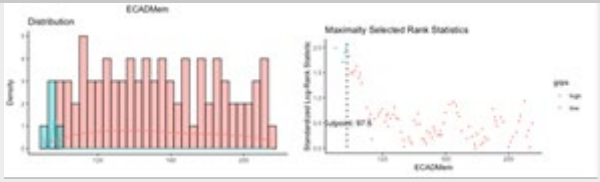
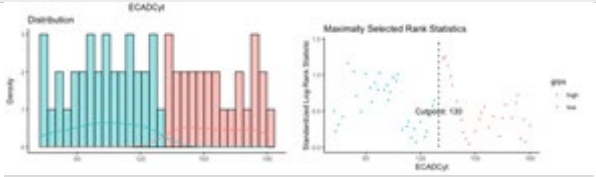
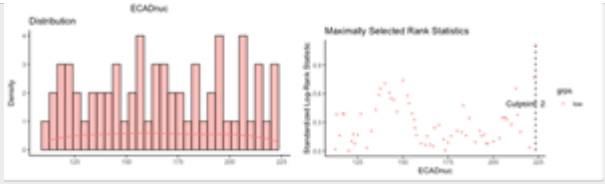
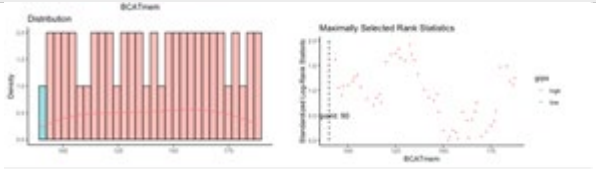
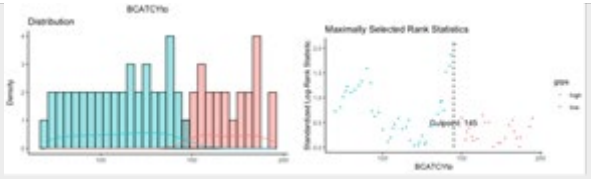
A detailed description has already been given in the Generic Methods chapter (section 2.6) regarding the immunohistochemical staining of the five markers of EMT was used in this paper and Figure 2.8 gives representative examples of low and high staining for each marker. Scoring of the stained slides was performed using the weighted histoscore, also detailed in the Generic Methods chapter (section 2.7).

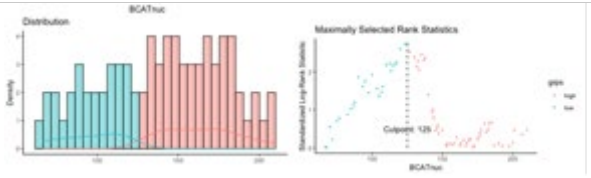
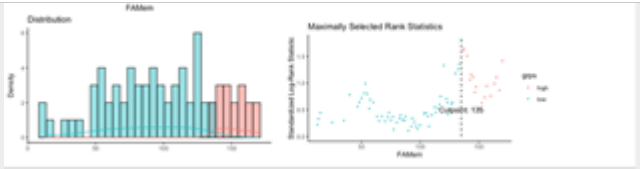
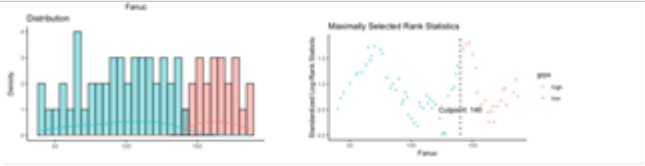
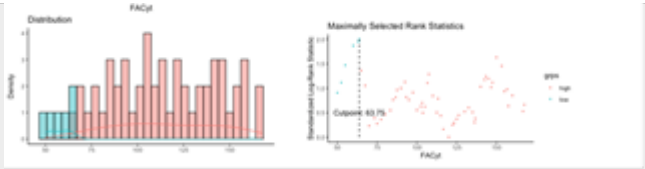
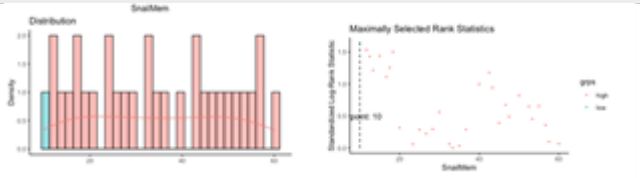
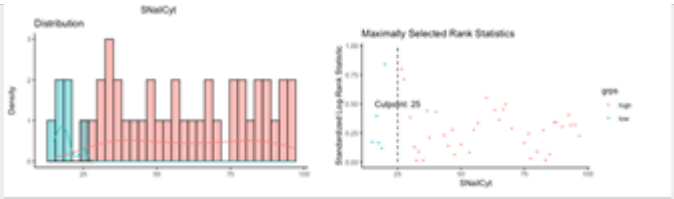
After staining, all slides were scanned using Hamamatsu NanoZoomer (Welwyn Garden City, Hertfordshire, UK) at 20x magnification and visualised on NDP viewer (NanoZoomer Digital Pathology software, Hamamatsu Photonics K.K.). EMT marker staining was assessed by Peter Alexander, blinded to clinicopathological data. Each marker was scored in tumour cell membrane, cytoplasm and nucleus. An average of the scored cores was taken as the final value. 192 cores were co-scored by Professor Joanne Edwards with excellent correlation (ICC >0.88 for all markers and loci).

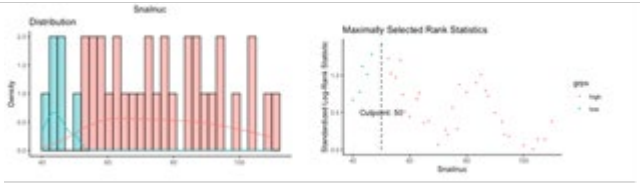
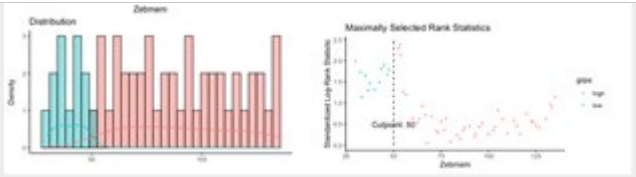
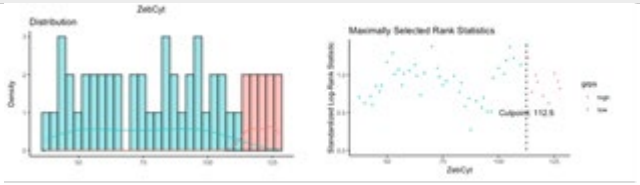
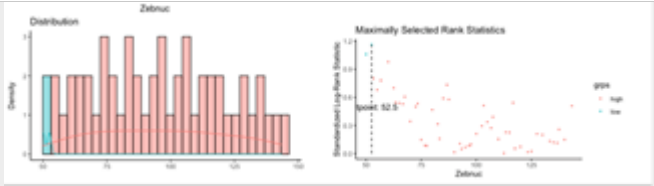
3.1.4 Statistical analysis

Data for EMT markers were dichotomised into high and low scores by Kathryn Pennel using a data-derived threshold for each score at each cellular location according to CSS using RStudio (R Studio, Inc, MA, USA), (Table 3.1). Missing data was excluded from analysis. All other data analysis was performed using SPSS version 27.0 (IBM SPSS). Univariate Cox regression analysis was used to calculate Hazard Ratios (HR) with 95% Confidence Intervals (CI) for CSS. When testing for associations between categorical variables, Pearson's chi-squared test was used. Where there were fewer than n=6 events in any cell, chi-squared analysis was not performed. A *p*-value of less than 0.05 was considered statistically significant.

Table 3.1. Median, Data-driven thresholds with graphs, range and number of tumours with high vs low expression for each EMT marker and cellular location (with thanks to Kathryn Pennel for providing these).

Marker Locus	**Median**	**Data-driven threshold**	**R-derived** threshold **graphs**	**Range**	**High, *N* (%)**	**Low *N* (%)**
E-cadherin Membrane	151.88	97.50		0 – 280	28 (12)	210 (88)
Cytoplasm	120.00	130.00		0 – 252.5	140 (59)	98 (41)
Nucleus	176.67	223.33		0 – 300	220 (92)	19 (8)
B-catenin Membrane	132.50	90.00		20 – 240	28 (12)	210 (88)
Cytoplasm	130.00	145.00		20 – 280	146 (61)	92 (39)

Nucleus	137.50	125.00		0 – 300	96 (40)	142 (60)
Fascin Membrane	100.00	135.00		0 – 300	171 (72)	67 (28)
Cytoplasm	110.00	63.75		0 – 300	31 (13)	207 (87)
Nucleus	120.00	140.00		0 – 300	159 (67)	79 (33)
Snail Membrane	25.00	10.00		0 – 187.5	46 (19)	192 (81)
Cytoplasm	47.50	25.00		0 – 160	56 (24)	182 (76)

Nucleus	70.00	50.00		0 – 200	55 (23)	183 (77)
Zeb-1 Membrane	70.00	50.00		10 – 235	74 (31)	164 (69)
Cytoplasm	80.00	112.50		10 – 210	195 (82)	43 (18)
Nucleus	90.00	52.50		0 – 200	26 (11)	212 (89)

3.2 RESULTS

There was a total of 502 patients undergoing potentially curative resection of Stage II-III CRC that also had a valid sample for assessment of one or more EMT markers and GMS, but only 238 tumours had scores for all five markers of EMT. Clinicopathological characteristics are given for patients with full scores available vs patients with missing scores in Table 3.2. There were no significant differences for any clinicopathological characteristic between these two groups. For those patients with scores available, 57% of patients were younger than 75 years, whereas 38% were node-positive. Fifty-three percent had low-risk disease, while 47% had high-risk disease. The medians, range and split into high and low for each marker are presented in Table 3.1. Median follow up for survivors was 140 months (interquartile range: 120 – 175). There were 156 deaths, of which 61 were CRC-related.

Table 3.2. Clinicopathological data for patients with valid GMS and EMT scores in TNM II-III disease (*N*=238) vs those without valid scores available (*N*=264)

Clinicopathological characteristics	All scores available *N* (%)[a]	Scores missing *N* (%)[a]	X^2
Age			
≤64	60 (25)	72 (27)	0.41
65-74	76 (32)	89 (34)	
≥75	102 (43)	264 (39)	
Gender			
Female	127 (53)	126 (48)	0.21
Male	111 (47)	138 (52)	
Presentation			
Elective	168 (71)	180 (68)	0.56
Emergency	70 (29)	84 (32)	
TNM			
I-II (low-risk)	127 (53)	128 (49)	0.28
II-III (high-risk)	111 (47)	136 (51)	
T-stage			
I	1 (1)	3 (1)	0.37
II	8 (3)	4 (4)	
III	159 (67)	169 (64)	
IV	70 (29)	88 (33)	
N-stage			
0	147 (62)	156 (59)	0.85
I	60 (25)	76 (29)	
II	31 (13)	31 (12)	
Site			
Colon	201 (85)	223 (85)	0.99
Rectum	37 (15)	41 (15)	
Differentiation			
Well/mod	206 (87)	239 (91)	0.16
Poor	32 (13)	25 (9)	
Venous invasion			
Absent	156 (66)	175 (66)	0.86
Present	82 (34)	89 (34)	
Tumour budding			
Present	173 (73)	203 (77)	0.28
Absent	65 (27)	61 (23)	
MMR			
Proficient	192 (81)	211 (81)	0.96
Deficient	46 (19)	50 (19)	
Tumour perforation			
No	221 (93)	245 (93)	0.98
Yes	17 (7)	19 (7)	
Peritoneal involvement			
No	167 (70)	184 (70)	0.91
Yes	71 (30)	80 (30)	
GMS			
0	61 (26)	82 (31)	0.07
1	133 (56)	147 (56)	
2	44 (18)	35 (13)	
Modified GPS			
0	83 (53)	100 (46)	0.51
1	37 (24)	71 (33)	
2	35 (23)	45 (21)	

[a]percentages rounded to nearest whole number and may not total 100%
Bold indicates significant result

Univariate CSS was assessed for each EMT marker (Table 3.3, Figure 3.1). E-cadherin, Fascin and Snail did not associate with survival at any cellular locus. Cytoplasmic and nuclear B-catenin were significant for worse CSS (HR 1.67, 95% CI 1.01-2.76, $p<0.05$, and HR 2.22, 95% CI 1.24-3.97, $p<0.01$, respectively). Membrane Zeb-1 was also significant for worse CSS (HR 2.00, 95% CI 1.07-3.77, $p=0.03$).

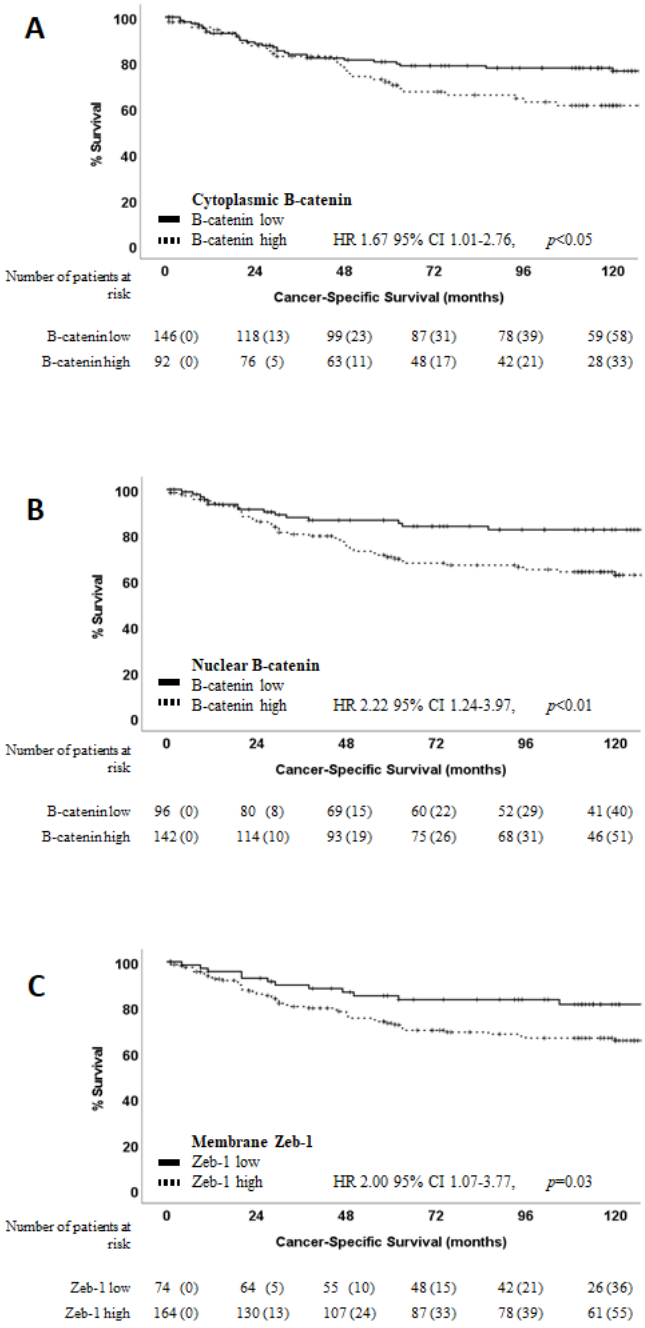


Figure 3.1. Cancer-specific survival for (A) Cytoplasmic and (B) Nuclear B-catenin, and (C) Membrane Zeb-1, in stage II-III CRC (n=238).

Table 3.3. Cancer-specific survival in stage II-III colorectal cancer for individual EMT markers (*N*=238).

Clinicopathological characteristics		**Cancer-specific survival**		
	N (%)[a]	Events (CSS)	Univariate HR (95% CI)	*P*
E-Cadherin				
Membrane Low	28 (12)	3		
Membrane High	210 (88)	58	2.80 (0.88-8.94)	0.08
Cytoplasm Low	140 (59)	30		
Cytoplasm High	98 (41)	31	1.40 (0.85-2.32)	0.19
Nucleus Low	220 (92)	57		
Nucleus High	18 (8)	4	0.71 (0.26-1.94)	0.50
B-Catenin				
Membrane Low	28 (12)	3		
Membrane High	210 (88)	58	2.92 (0.92-9.34)	0.07
Cytoplasm Low	146 (61)	30		
Cytoplasm High	92 (39)	31	1.67 (1.01-2.76)	**0.046**
Nucleus Low	96 (40)	15		
Nucleus High	142 (60)	46	2.22 (1.24-3.97)	**0.007**
Fascin				
Membrane Low	171 (72)	40		
Membrane High	67 (28)	21	1.62 (0.96-2.75)	0.07
Cytoplasm Low	31 (13)	5		
Cytoplasm High	207 (87)	56	1.91 (0.76-4.76)	0.17
Nucleus Low	159 (67)	37		
Nucleus High	79 (33)	24	1.53 (0.92-2.57)	0.10
Snail				
Membrane Low	46 (19)	16		
Membrane High	192 (81)	45	0.60 (0.34-1.07)	0.08
Cytoplasm Low	56 (24)	18		
Cytoplasm High	182 (76)	43	0.72 (0.42-1.26)	0.25
Nucleus Low	55 (23)	11		
Nucleus High	183 (77)	50	1.46 (0.76-2.80)	0.26
Zeb1				
Membrane Low	74 (31)	12		
Membrane High	164 (69)	49	2.00 (1.07-3.77)	**0.03**
Cytoplasm Low	195 (82)	53		
Cytoplasm High	43 (18)	8	0.64 (0.31-1.35)	0.24
Nucleus Low	26 (11)	5		
Nucleus High	212 (89)	56	1.60 (0.64-3.98)	0.32

EMT score (original)				
Absent EMT	7 (3)	1	1.94 (0.27-14.00)	0.51
Low EMT	231 (97)	60	REF	1.0
High EMT	0 (0)	-	-	-

[a]percentages rounded to nearest whole number and may not total 100%

Bold indicates significant result

In terms of associations between EMT markers and clinicopathological variables, these are given in Tables A2.1 to A2.5. In brief, E-cadherin did not associate with any clinicopathological variables.

Nuclear B-catenin was more likely to be found in rectal tumours ($p=0.03$), cytoplasmic and nuclear B-catenin were associated with well/moderate differentiation ($p=0.03$ and $p<0.01$), B-catenin at any cellular location was associated with MMR proficiency (all $p<0.001$), and membrane B-catenin was associated with lower peritoneal involvement ($p=0.04$).

Nuclear Fascin associated with poorer differentiation ($p=0.03$), while membrane Fascin associated with greater peritoneal involvement ($p=0.03$).

Snail did not associate with any clinicopathological variables.

Cytoplasmic Zeb-1 associated with lower venous invasion ($p=0.04$).

There was no association between any EMT marker and tumour budding or lymph node status.

In a previous study, a combined EMT score was constructed(Roseweir et al., 2019), which divided patients into three groups as follows: absent EMT described high membrane E-cadherin with all other markers low; low EMT was marked by low membrane E-cadherin or high individual markers; high EMT was marked by low membrane E-cadherin and all other markers high. However, due to differences in staining between the original study and the present study, the data thresholds from the original study could not be used. New thresholds were thus generated using R Studio as displayed in Table 3.1. Once these thresholds were applied to the present cohort, there were no tumours identified as having "high EMT" and only 7 with absent EMT (Table 3.3). The combined EMT score was therefore not employed in this study.

Table 3.4. Associations of EMT markers with GMS in stage I-III colorectal cancer

	GMS category						
	0 (n=61) *N* (%)[a]		**1** (n=133) *N* (%)		**2** (n=44) *N* (%)		*Pearson* X^2
E-Cadherin							
Membrane Low	9	(15)	12	(9)	7	(16)	1.00
Membrane High	52	(85)	121	(91)	37	(84)	
Cytoplasm Low	38	(62)	76	(57)	26	(59)	0.69
Cytoplasm High	23	(38)	57	(43)	18	(41)	
Nucleus Low	55	(90)	122	(92)	43	(98)	-[b]
Nucleus High	6	(10)	11	(8)	1	(2)	
B-Catenin							
Membrane Low	12	(20)	10	(8)	6	(14)	0.22
Membrane High	49	(80)	123	(92)	38	(86)	
Cytoplasm Low	36	(59)	81	(61)	29	(66)	0.49
Cytoplasm High	25	(41)	52	(39)	15	(34)	
Nucleus Low	32	(53)	50	(38)	14	(32)	**0.03**
Nucleus High	29	(47)	83	(62)	30	(68)	
Fascin							
Membrane Low	50	(82)	89	(67)	32	(73)	0.21
Membrane High	11	(18)	44	(33)	12	(27)	
Cytoplasm Low	10	(16)	12	(9)	9	(21)	0.72
Cytoplasm High	51	(84)	121	(91)	35	(79)	
Nucleus Low	43	(71)	87	(65)	29	(66)	0.58
Nucleus High	18	(29)	46	(35)	15	(34)	
Snail							
Membrane Low	11	(18)	23	(17)	12	(27)	0.29
Membrane High	50	(82)	110	(83)	32	(73)	
Cytoplasm Low	17	(28)	27	(20)	12	(27)	0.82
Cytoplasm High	44	(72)	106	(80)	32	(73)	
Nucleus Low	13	(21)	28	(21)	14	(32)	0.25
Nucleus High	48	(79)	105	(79)	30	(68)	
Zeb1							
Membrane Low	17	(28)	43	(32)	14	(32)	0.63
Membrane High	44	(72)	90	(68)	30	(68)	
Cytoplasm Low	47	(77)	109	(82)	39	(89)	-[b]
Cytoplasm High	14	(23)	24	(18)	5	(11)	
Nucleus Low	9	(15)	14	(11)	3	(7)	-[b]
Nucleus High	52	(85)	119	(89)	41	(93)	

[a]percentages rounded to nearest whole number and may not total 100%

[b]for cells where n<6, *Pearson* X^2 analysis was not performed. **Bold** indicates significant result

Associations between individual EMT markers and GMS were subsequently assessed (Table 3.4). Nuclear B-catenin was the only EMT marker with a significant association with GMS as a whole (p=0.03). For GMS 2 tumours, 68% had high nuclear B-catenin vs 47% for GMS 0, in keeping with EMT as a key process in mesenchymal tumours. However, GMS 0, 1 and 2 are not associated linearly but are in fact separate entities categorised by phenotypic microscopic appearance. Therefore, the phenotypic elements that comprise GMS (i.e., KM and TSP) were assessed individually for associations with markers of EMT (Table 3.5). The analysis for strong KM vs weak KM revealed that membrane B-catenin was significantly lower in strong KM (p=0.03). Nuclear B-catenin was again demonstrated as significantly lower in strong KM (p=0.03). Membrane Fascin was also significantly lower in strong KM (p=0.04). Membrane Fascin was highest in GMS 1 and slightly lower in GMS 2, hence why there was no linear association with GMS as a whole (Table 3.4). There were no other associations between GMS categories and EMT markers, neither were there any further associations between TSP and markers of EMT.

Table 3.5. Associations of EMT markers according to pathological phenotype in stage II-III colorectal cancer

	Immune phenotype (KM)					**Mesenchymal phenotype (TSP)**				
	KM strong (n=61) *N* (%)[a]		**KM weak** (n=177) *N* (%)		*Pearson* X^2	**TSP low** (n=183) *N* (%)		**TSP high** (n=55) *N* (%)		*Pearson* X^2
E-Cadherin										
Membrane Low	9	(11)	19	(15)	0.40	21	(12)	7	(13)	0.80
Membrane High	52	(89)	158	(85)		162	(89)	48	(87)	
Cytoplasm Low	38	(62)	102	(58)	0.52	107	(59)	33	(60)	0.84
Cytoplasm High	23	(38)	75	(42)		76	(42)	22	(40)	
Nucleus Low	55	(90)	165	(93)	0.44	166	(91)	54	(98)	-[b]
Nucleus High	6	(10)	12	(7)		17	(9)	1	(2)	
B-Catenin										
Membrane Low	12	(20)	16	(9)	**0.03**	22	(12)	6	(11)	0.82
Membrane High	49	(80)	161	(91)		161	(88)	49	(89)	
Cytoplasm Low	36	(59)	110	(62)	0.67	113	(62)	33	(60)	0.82
Cytoplasm High	25	(41)	67	(38)		70	(38)	22	(40)	
Nucleus Low	32	(53)	64	(36)	**0.03**	79	(43)	17	(31)	0.10
Nucleus High	29	(47)	113	(64)		104	(57)	38	(69)	
Fascin										
Membrane Low	50	(82)	121	(68)	**0.04**	132	(72)	39	(71)	0.86
Membrane High	11	(18)	56	(32)		51	(28)	16	(29)	
Cytoplasm Low	10	(16)	21	(12)	0.37	22	(12)	9	(16)	0.40
Cytoplasm High	51	(84)	156	(88)		161	(88)	46	(84)	
Nucleus Low	43	(71)	116	(66)	0.89	126	(69)	33	(60)	0.22
Nucleus High	18	(29)	61	(35)		57	(31)	22	(40)	
Snail										
Membrane Low	11	(18)	35	(20)	0.77	31	(17)	15	(27)	0.09
Membrane High	50	(82)	142	(80)		152	(83)	40	(40)	
Cytoplasm Low	17	(28)	39	(22)	0.36	41	(22)	15	(27)	0.46
Cytoplasm High	44	(72)	138	(78)		142	(78)	40	(73)	
Nucleus Low	13	(21)	42	(24)	0.70	40	(22)	15	(27)	0.41
Nucleus High	48	(79)	135	(76)		143	(78)	40	(73)	
Zeb1										
Membrane Low	17	(28)	57	(32)	0.53	56	(31)	18	(33)	0.77
Membrane High	44	(72)	120	(68)		127	(69)	37	(67)	
Cytoplasm Low	47	(77)	148	(84)	0.25	148	(81)	47	(86)	0.44
Cytoplasm High	14	(23)	29	(16)		35	(19)	8	(14)	
Nucleus Low	9	(15)	17	(10)	0.27	23	(13)	3	(6)	-[b]
Nucleus High	52	(85)	160	(90)		160	(87)	52	(95)	

[a]percentages rounded to nearest whole number and may not total 100%

[b]for cells where n<6, *Pearson* X^2 analysis was not performed

Bold indicates significant result

3.3 DISCUSSION

The results displayed once again demonstrate the association between B-catenin and survival in stage II-III CRC. Both cytoplasmic and nuclear B-catenin were associated with poor survival outcome. Whereas in the present study the higher expression of cytoplasmic and nuclear B-catenin was associated with worse survival outcome, Roseweir et al.(Roseweir et al., 2019) found the loss of membrane B-catenin to have the same effect.

Furthermore, the presence of membrane Zeb-1 was found to be significant for CSS. Others have found the presence of Zeb-1 to be associated with a process known as "vasculogenic mimicry", the ability of cells to express endothelial cell markers, which is thought to be a feature of EMT(Liu et al., 2012). In a murine model Kudo et al.(Kudo et al., 2015) found that CRP suppressed Zeb-1 on colon cancer tumour cells. CRP is one of the main markers of the modified Glasgow Prognostic Score (mGPS), which identifies individuals with systemic inflammation and is a known poor prognostic indicator(Park et al., 2016b). However, in the present study there was no association between serum CRP and Zeb-1 expression at any cellular locus (data not shown). The presence of Zeb-1 when assessed by realtime PCR in colorectal cancers has also previously been demonstrated to indicate worse survival, independent of other clinicopathological features on multivariate analysis(Zhang et al., 2013).

The aforementioned combined EMT score was not able to split the patients adequately into the three different stages of EMT as in the original study(Roseweir et al., 2019) and the reasons for this are unclear. Different data thresholds were necessary and this may have had an influence. The reason that different thresholds were required in this study is that the specimens stained in the previous study had globally lower weighted histoscores. Since the staining technique was standardised and the antibodies used were the same antibodies from the same suppliers, it is unclear why this was the case. It is possible that batch-to-batch variability of antibodies or a difference in production by the company, for example, of the

secondary DAB stain, caused this change in staining density. Therefore, it is not possible to say whether the thresholds set in the current study will be applicable to future studies. Perhaps the inability for the original score to apply is due to differences in the patient cohorts studied, although they were of similar TNM stage. It may be that the difference lies in the patients with missing scores although the numbers for the original study and the present study are similar and the clinicopathological variables for patients with missing scores were not statistically different from those with a complete set of scores available.

The relationship between individual EMT markers and GMS was assessed. GMS 0, the CRC phenotype characterised by strong peritumoural inflammation (KM), was found to be associated with lower membrane Fascin expression. Fascin over-expression has been implicated in chronic inflammation-related colorectal cancer carcinogenesis(Kanda et al., 2018). Conversely, strong peritumoural inflammation in the context of CRC as a whole, is known to be a good prognostic indicator(Alexander et al., 2020b) and appears to be protective against mesenchymal phenotype(Park et al., 2015, Li et al., 2018a). Therefore, the presence of lower membrane Fascin expression in GMS 0 may be reflective of a less aggressive phenotype than the other two GMS categories. Furthermore, membrane and nuclear expression of B-catenin was significantly lower in GMS 0. The loss of membrane B-catenin is believed to occur simultaneously with the loss of membrane E-cadherin and is one of the hallmarks of EMT(Brabletz et al., 2005). The data demonstrates that this group, while categorised by the protective feature of strong peritumoural inflammation, have a lower membrane expression of B-catenin than tumours with weak peritumoural inflammation, a feature that Roseweir et al.(Roseweir et al., 2019) found to predict worse outcome. Briede et al.(Briede et al., 2020) recently published a study on a Latvian CRC cohort finding no association with peritumoural inflammation and E-cadherin, but did not assess any other markers of EMT. Zlobec et al.(Zlobec et al., 2007), on the other hand, found peritumoural inflammation to be protective against the otherwise negative feature of E-

cadherin loss. These studies both used E-cadherin as a primary marker of EMT. No other studies were identified assessing peritumoural inflammation in the context of other markers of EMT. It is unclear why there would be lower expression of membrane B-catenin in this group. However, it may be that, whilst not directly linked, the strong peritumoural inflammation is protective in spite of loss of membrane B-catenin in this subgroup.

GMS 1 defines a CRC phenotype with neither strong peritumoural inflammation, nor high TSP and believed to represent CMS 2 and 3. This phenotype was previously demonstrated to have an intermediate survival outcome compared with the other two GMS categories(Park et al., 2015, Alexander et al., 2020c). GMS 1 tumours were observed to have the highest membrane and cytoplasmic Fascin. Due to Fascin's role in bundling the cell's actin cytoskeleton and the greater motility of cells with higher levels of Fascin(Vignjevic et al., 2007), this may indicate that some of these tumours already have features of EMT, although they do not display the phenotype of higher TSP.

In GMS 2, the CRC phenotype characterised by high TSP and worse survival, there were greater numbers of tumours with high nuclear B-catenin, in keeping with a mesenchymal phenotype. However, cytoplasmic Fascin levels were lower in this group and the role of cytoplasmic Fascin is therefore unclear. Perhaps the role of Fascin in the EMT process is in facilitating transition to the mesenchymal phenotype and it may play a lesser role once this phenotype has been attained. This feature also requires further investigation in independent cohorts. There were fewer associations between EMT markers and GMS 2 than originally anticipated. This may reflect the fact that high tumour stroma may not in fact indicate evidence of EMT, but instead reflect the ability of the tumour to recruit the hosts local fibroblasts and transform these by a variety of chemokines to enable the negative pathological features associated with high tumour stroma, such as neoangiogenesis(Conti and Thomas, 2011, McCorry et al., 2018). Furthermore, Menezes et al.(Menezes et al., 2022) have described a plasticity and adaptability in the different subpopulations of cancer

associated fibroblasts, which are able to convert from myofibroblasts to inflammatory fibroblasts. They discuss these with regard to the tumour's ability to resist chemotherapy, but the different subpopulations of cancer associated fibroblasts illustrate the complex nature of the interactions between tumour and host.

In conclusion, the data presented confirm the prognostic significance of markers of EMT in CRC that have been identified in previous studies, in particular B-catenin and membrane Zeb-1. Furthermore, markers of EMT have been demonstrated to associate with individual GMS categories in a manner not previously identified. Specifically, nuclear B-catenin levels increased with increasing GMS category; membrane Fascin levels similarly were lowest in GMS 0 and highest in GMS 1, which may indicate an early role in transition to the mesenchymal phenotype that is less pronounced after this phenotypic appearance has been achieved. These findings warrant further investigation in independent patient cohorts.

The significance of GMS was therefore demonstrated in relation to other known prognostic biomarkers in CRC.

4. GMS VALIDATION

Attention was next turned to the validating the GMS in additional patient cohorts. Until the present research project, GMS had been reported only in the patients of Glasgow Royal Infirmary represented by the JP TMA(Park et al., 2015).

Four cohorts were selected for further study of GMS. The first was the AP TMA (presented as a poster at ASCO GI in 2020(Alexander et al., 2020a). Secondly, the GRI-CRC-TMA cohort was used to assess interesting findings in colon cancers identified in the AP TMA cohort. Thirdly, the combined JP-AP TMA was assessed as an expanded cohort to enable the role of GMS in subgroup analysis. Finally, the TransScot cohort, subject to less bias data were collected within the context of a clinical trial, was used to validate the GMS. The data for these latter two cohorts were published together in the BJC(Alexander et al., 2020c).

4.1 SPECIFIC METHODS

4.1.1 Patient cohorts

The AP TMA was used to initially validate GMS in an independent cohort of CRC patients. Four-hundred and ninety-five TNM II-III colon cancer specimens were identified retrospectively from this database All patients had undergone surgery with curative intent between 2000 and 2007. Those who had endoscopic or palliative procedures and those with involved surgical margins (R1) were excluded. The primary endpoints were cancer-specific survival (CSS; defined as time from surgery to death from colon cancer) and overall survival (OS; measured from date of surgery until all-cause mortality). Survival data were available until the 1st July 2020.

The GRI-CRC-TMA dataset: the patients in this cohort were derived from a previously published cohort of one thousand patients having colorectal cancers resected between January 1997 and May 2013 in Glasgow Royal Infirmary(Park et al., 2016b). However, specimens were only available for 849 cases. The following exclusions were applied: thirty-

day mortality, rectal cancer, TNM 4 disease, and R1 resection (positive resection margins). Of the patients with samples available, there were 554 remaining following exclusions. The primary endpoints were cancer-specific survival (CSS; defined as time from surgery to death from colon cancer) and overall survival (OS; measured from date of surgery until all-cause mortality). Survival data were available until the 1st July 2020.

The combined JP-AP TMA was used to create an expanded validation cohort. The combined JP-AP cohort was chosen for publication as the increased numbers enabled further evaluation of T-stage and N-stage by GMS subgroup. This cohort included 862 TNM I-III CRC, combining individuals from the discovery Glasgow Royal Infirmary cohort (*n*=231) with additional patients identified retrospectively from other Glasgow hospitals (Western Infirmary, Gartnavel General and Stobhill Hospitals) who had undergone surgery with curative intent from 2000-2007 (*n*=631). Those who had endoscopic or palliative procedures and those with involved surgical margins (R1) were excluded. The primary endpoint was disease-free survival (DFS; measured from date of surgery/randomization to date of recurrence or all-cause mortality) for this cohort, in order to aid comparison with the TransScot cohort. In addition, relapse-free survival (RFS; measured from date of surgery to date of recurrence or CRC-related mortality), cancer-specific survival (CSS; measured from date of surgery until CRC-related mortality) and overall survival (OS; measured from date of surgery until all-cause mortality) were calculated. Survival data was complete up until 9th February 2017 for this cohort, which functioned as censor date.

The TransScot cohort comprised 2912 patients with available tissue from the SCOT adjuvant chemotherapy trial (ISRCTN no. 59757862) who had undergone potentially curative resection for high-risk TNM II or TNM III CRC from 2008-2013 within the UK. All patients were followed up for at least 3 years. The primary endpoint was disease-free survival (DFS; measured from date of surgery/randomization to date of recurrence or all-cause mortality)

for this cohort. DFS was the only form of survival data available for study in this cohort. Survival data was complete up until the end of the study period for the TransSCOT cohort. Those who died within 30 days of surgery were excluded from all cohorts.

4.1.2 Clinicopathological characteristics

Tumour budding(van Wyk et al., 2019) and MMR status(Powell, 2016) were already available for this cohort. The Petersen index was used to assess clinical risk as in clinical practice indicating low or high-risk stage II colorectal cancer(Petersen et al., 2002): venous invasion and peritoneal involvement were assigned a score of 1, while tumour perforation was assigned a score of 2. TNM II disease with Petersen index of 2 or higher, or TNM III disease was considered high-risk. Peritumoural inflammatory scores (KM grade) and tumour stromal percentage scores (TSP) were already available(van Wyk et al., 2019). These were combined as the GMS as previously described(Alexander et al., 2020c). The Methods Chapter also outlines the technique in full (sections 2.4.1 and 2.4.2). The modified Glasgow Prognostic score (mGPS) was calculated using serum CRP (C-reactive protein) and albumin levels obtained in the 30 days before surgery or at the time of admission as previously described(Park et al., 2016b).

4.1.3 Immunohistochemical staining

Immunohistochemistry for generic T-cell (CD3) and cytotoxic (CD8) T-cell densities within the invasive margin, tumour stroma and cancer cell nests had previously been performed and reported for the JP TMA(Richards et al., 2014). In addition, a composite CD3/CD8 score comprising respective densities in the tumour centre and invasive margin was calculated, ranging from 0 (both CD3 and CD8 low in both regions) to 4 (both high in both regions).

4.1.4 Mutational analysis

Mutational analysis was performed on a subset of patients from the combined JP-AP TMA (n=251). DNA was extracted from FFPE sections by NHS Tayside diagnostics and stored at -80ºC. DNA concentration was determined using Qubit assays (Thermo Fisher Scientific,

MA, USA) and samples with ≥150ng DNA were included in the study. DNA was diluted to 4ng/µl and transferred to barcoded library tubes. Sequencing was performed by the Glasgow Precision Oncology Laboratory (GPOL) using the GPOL 151 CORE Cancer gene panel and run on a HiSeq4000 (Illumina, CA, USA). Data for KRAS and BRAF were converted to mutation annotation format and analysed using BiocManager maftools package in RStudio (R Studio, Inc, MA, USA).

4.1.5 Statistical analysis

All data analysis was performed using SPSS version 28.0 (IBM SPSS). Kaplan-Meier and log-rank analysis compared survival adjusted for T-stage, N-stage and treatment duration, where appropriate. Hazard ratios (HR) and confidence intervals (CI) were calculated from univariate Cox regression survival analysis. Multivariable survival analysis using a backward conditional elimination model and a statistical significance threshold of *p*-value<0.1 was performed to identify independent prognostic biomarkers. Text results are reported as HR, 95% CI for GMS 0 vs GMS 2, but p-value given is for log-rank analysis of overall trend. Pearson chi-squared test was used to test associations between categorical variables and GMS.

4.2 VALIDATING THE GMS IN THE AP TMA

4.2.1 Results of survival analysis in the AP TMA cohort

In the AP TMA cohort, there were 495 patients with stage I-III colon cancer and a valid GMS. Clinicopathological characteristics are presented in table 4.1. Fifty-seven percent of patients were younger than 75 at the time of surgery and 36% were node-positive. One hundred and forty-seven patients (30%) were GMS of 0, 277 patients (56%) were GMS 1 and 71 patients (14%) were GMS 2. The median length of follow up of survivors was 11.8 years (Interquartile range: 10.0-13.75). During follow up there were 310 deaths, of which 125 were colon cancer-related. Five-year CSS was 77% across the cohort. For stages I, II and III, respectively, 5-year CSS rates were 98%, 82% and 62%.

Table 4.1. Clinicopathological characteristics and their relation to GMS in patients undergoing curative resection for colon cancer (AP TMA)

Clinicopathological characteristics			**GMS**						
	N (%)[a]		**0** (n=147) *N*	(%)	**1** (n=277) *N*	(%)	**2** (n=71) *N*	(%)	*P*
Age									
<65	125	(25)	35	(24)	62	(22)	28	(39)	**0.03**
65-74	158	(32)	44	(30)	93	(34)	21	(30)	
>74	212	(43)	68	(46)	122	(44)	22	(31)	
Gender									
Female	245	(49)	79	(54)	136	(49)	35	(49)	0.44
Male	250	(51)	68	(46)	141	(51)	36	(51)	
Presentation									
Elective	341	(69)	117	(80)	179	(65)	45	(63)	**0.003**
Emergency	154	(31)	30	(20)	98	(35)	26	(37)	
TNM									
I-II	321	(65)	112	(76)	175	(63)	34	(48)	**<0.001**
III	174	(35)	35	(24)	102	(37)	37	(52)	
T-stage									
1	21	(4)	16	(11)	5	(2)	0		**<0.001**
2	53	(11)	28	(19)	20	(7)	5	(7)	
3	279	(56)	74	(50)	167	(60)	38	(54)	
4	142	(29)	29	(20)	85	(31)	28	(39)	
N-stage									
0	320	(65)	112	(76)	174	(63)	34	(48)	**<0.001**
1	117	(24)	26	(18)	67	(24)	24	(34)	
2	57	(12)	9	(6)	35	(13)	13	(18)	
Differentiation									
Well/mod	444	(90)	133	(90)	245	(88)	66	(93)	0.79
Poor	51	(10)	14	(10)	32	(12)	5	(7)	
Venous invasion									
Absent	341	(69)	116	(79)	189	(68)	36	(51)	**<0.001**
Present	154	(31)	31	(21)	88	(32)	35	(49)	
Tumour budding									
Present	123	(25)	41	(28)	64	(23)	18	(25)	0.51
Absent	372	(75)	106	(72)	213	(77)	53	(75)	
MMR									
Proficient	391	(79)	104	(71)	227	(82)	60	(86)	**0.003**
Deficient	102	(21)	43	(29)	49	(18)	10	(14)	

[a]percentages rounded to nearest whole number – may not total 100%. Bold indicates $p<0.05$

Associations between GMS and CSS were assessed (Table 4.2). CSS was stratified by GMS in the whole cohort with 5-year CSS of 89%, 74% and 66% for GMS 0, 1 and 2, respectively (GMS 0 vs GMS 2: HR 3.12 95% CI 1.74-5.58, $p<0.001$; Figure 4.1A). On multivariate analysis for CSS, GMS remained independent (p=0.04) of emergency presentation (p<0.01), T-stage (p=0.03) and N-stage (p<0.001), (Table 4.3). Subgroup analysis was performed according to mode of presentation, node-negative/-positive disease, venous invasion and MMR-status (Table 4.2). GMS stratified CSS for elective presentation (GMS 0 vs GMS 2; HR 3.22 95% CI 1.47-7.05, $p=0.001$), but was unable to stratify CSS in emergency presentation. GMS stratified CSS for node-negative (GMS 0 vs GMS 2; HR 3.55 95% CI 1.62-7.79, $p=0.003$), (Figure 4.1B). However, for node-positive disease, the picture was somewhat complex, since the GMS 2 patients in this subgroup for this cohort did not follow the expected trajectory. Therefore, while the trend was not significant for GMS as a whole, CSS was nevertheless stratified in this subgroup for GMS 0 vs GMS 1 (HR 2.98 95% CI 1.35-6.59), evidencing the protective effect of strong KM (Figure 4.1C). GMS stratified CSS regardless of the presence of venous invasion (venous invasion absent: GMS 0 vs GMS 2; HR 2.31 95% CI 1.07-4.97, $p=0.01$; venous invasion present: GMS 0 vs GMS 2; HR 4.37 95% CI 1.45-13.18, $p<0.01$). Finally, GMS was able to stratify MMR-proficient tumours (GMS 0 vs GMS 2; HR 3.95 95% CI 1.91-8.16, p<0.001, Figure 4.1D), but was unable to stratify CSS in MMR-deficient disease (n=102), due to small sample size (Figure 4.1E).

Table 4.2. Cancer-specific survival for GMS according to mode of presentation, TNM stage, venous invasion and MMR status in patients undergoing curative resection for stage I-III colon cancer (AP TMA).

Group (GMS category)		**Survival**			
	N	5-year CSS (%; SE)	Events (*N*=123)	HR (95% CI)	*P*
Whole cohort				*Trend*	*<0.001*
0	147	89 (3)	20	1.0 (reference)	
1	277	74 (3)	77	2.33 (1.42-3.81)	**0.001**
2	71	66 (6)	26	3.12 (1.74-5.58)	**<0.001**
Elective presentation				*Trend*	*0.001*
0	117	92 (3)	12	1.0 (reference)	
1	179	78 (3)	43	2.55 (1.34-4.83)	**<0.01**
2	45	73 (7)	13	3.22 (1.47-7.05)	**<0.01**
Emergency presentation				*Trend*	*0.11*
0	30	76 (8)	8	1.0 (reference)	
1	98	64 (5)	34	1.49 (0.91-3.23)	0.31
2	26	53 (10)	13	2.05 (0.85-4.95)	0.11
Stage I-II (N 0)				*Trend*	*0.003*
0	112	91 (3)	13	1.0 (reference)	
1	175	87 (3)	28	1.48 (0.76-2.85)	0.25
2	34	64 (8)	12	3.55 (1.62-7.79)	**<0.01**
Stage III (N 1-2)				*Trend*	*0.17*
0	35	84 (7)	7	1.0 (reference)	
1	102	51 (5)	49	2.98 (1.35-6.59)	**<0.01**
2	37	69 (8)	14	2.11 (0.85-5.23)	0.11
Venous invasion absent				*Trend*	*0.01*
0	116	90 (3)	16	1.0 (reference)	
1	189	77 (3)	46	1.92 (1.09-3.39)	**0.03**
2	36	76 (7)	11	2.31 (1.07-4.97)	**0.03**
Venous invasion present				*Trend*	*<0.01*
0	31	89 (6)	4	1.0 (reference)	
1	88	66 (5)	31	3.52 (1.24-9.97)	**0.02**
2	35	56 (9)	15	4.37 (1.45-13.18)	**<0.01**
MMR-proficient				*Trend*	*<0.001*
0	104	91 (3)	11	1.0 (reference)	
1	227	71 (3)	71	3.48 (1.84-6.57)	**<0.001**
2	60	68 (6)	22	3.95 (1.91-8.16)	**<0.001**
MMR-deficient				*Trend*	*0.83*
0	43	84 (6)	9	1.0 (reference)	
1	49	86 (5)	6	0.61 (0.22-1.72)	0.35
2	10	63 (17)	3	1.90 (0.51-7.05)	0.34

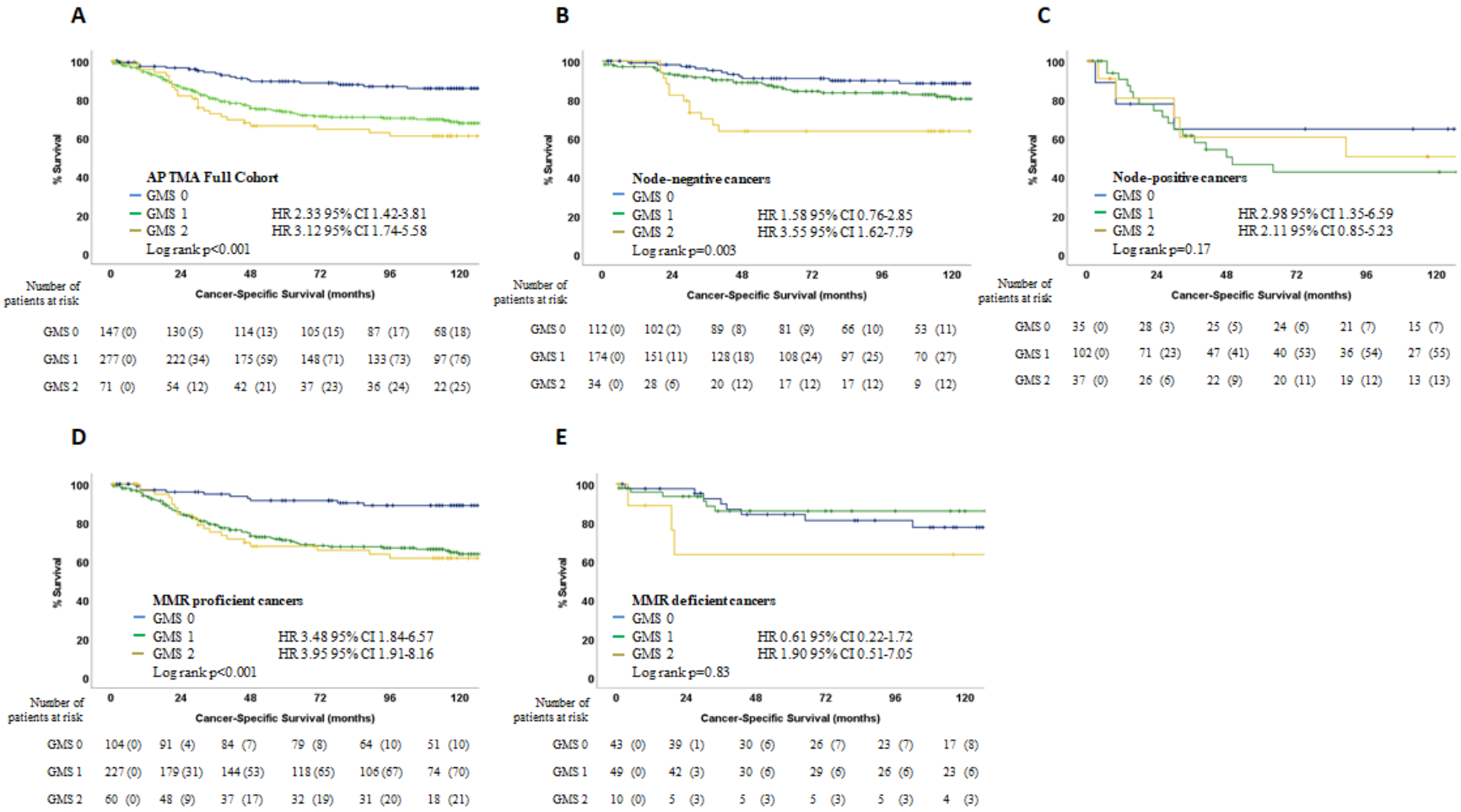


Figure 4.1. GMS stratification of CSS according to nodal and MMR status in stage I-III colon cancer (AP TMA). (A) Full cohort (n=495), (B) Node-negative (n=320), (C) Node-positive (n=174), (D) MMR-proficient (n-391) and (E) MMR deficient (n=102).

Table 4.3. Survival analysis for clinicopathological characteristics in patients undergoing curative resection for stage I-III colon cancer (AP TMA).

Clinicopathological characteristics			**Cancer-specific survival**			
	N (%)[a]		Univariate HR (95% CI)	*P*	Multivariate HR (95% CI)	*P*
Age						
<65	125	(25)				
65-74	158	(32)				
>74	212	(43)	1.12 (0.90-1.40)	0.31		
Gender						
Female	245	(49)				
Male	250	(51)	1.25 (0.88-1.77)	0.22		
Presentation						
Elective	341	(69)				
Emergency	154	(31)	2.34 (1.64-3.23)	**<0.001**	1.67 (1.15-2.43)	**<0.01**
TNM						
I-II	321	(65)				
III	174	(35)	2.67 (1.96-3.62)	**<0.001**[b]		
T-stage						
1	21	(4)				
2	53	(11)				
3	279	(56)				
4	142	(29)	2.28 (1.71-3.04)	**<0.001**	1.97 (1.06-3.68)	**0.03**
N-stage						
0	320	(65)				
1	117	(24)				
2	57	(12)	1.95 (1.57-2.43)	**<0.001**	1.66 (1.31-2.09)	**<0.001**
Differentiation						
Well/mod	444	(90)				
Poor	51	(10)	1.78 (1.07-2.98)	**0.03**	–	0.31
Venous invasion						
Absent	341	(69)				
Present	154	(31)	1.63 (1.14-2.34)	**<0.01**	–	0.52
Tumour budding						
Present	123	(25)				
Absent	372	(75)	1.37 (0.94-2.01)	0.11		
MMR						
Proficient	391	(79)				
Deficient	102	(21)	0.66 (0.40-1.09)	0.11		
Tumour perforation						
No	464	(94)				
Yes	31	(6)	1.38 (1.04-1.85)	**0.03**	–	0.48
Lymph node yield						
≥12	304	(61)				
<12	191	(39)	1.35 (0.95-1.92)	0.10		
Peritoneal involvement						
No	360	(73)				
Yes	135	(27)	2.45 (1.72-3.48)	**<0.001**	–	0.58
GMS						
0	147	(30)				
1	277	(56)				
2	71	(14)	1.73 (1.33-2.26)	**<0.001**	1.35 (1.02-1.79)	**0.04**

[a]percentages rounded to nearest whole number and may not total 100%

[b]not included in multivariate model as T-stage and N-stage included separately

Bold indicates $p<0.05$

Next, associations between GMS and OS were assessed (Table 4.4). OS was stratified by GMS in the whole cohort with 5-year CSS of 78%, 58% and 56% for GMS 0, 1 and 2, respectively and while GMS 0 vs GMS 2 was not significant (*p*0.06), there was a significant difference for GMS 0 vs GMS 1 (HR 1.46 95% CI 1.12-1.90, *p*=0.006; Figure 4.2A). On multivariate analysis for OS, GMS remained independent (p=0.02) of age (p<0.001), emergency presentation (p<0.001), N-stage (p<0.001) and tumour differentiation (p=0.04) (Table 4.5). Subgroup analysis was performed according to mode of presentation, node-negative/-positive disease, venous invasion and MMR-status (Table 4.4). Whilst GMS stratified OS for elective presentation as a whole, GMS 0 vs GMS 1 was significant as for the whole cohort (HR 1.47 95% CI 1.03-2.03, *p*=0.02). GMS was also unable to stratify OS in emergency presentation(*p*=0.81). GMS was unable to stratify OS for node-negative disease (*p*=0.50, Figure 4.2B). However, for node-positive disease, as for the whole cohort and elective cancer, GMS 0 vs GMS 2 was not significant (*p*=0.81), while GMS 0 vs GMS 1 was significant (HR 2.07 95% CI 1.20-3.5, *p*=0.009; Figure 4.2C). GMS was unable to stratify OS when venous invasion was absent (*p*=0.50) but was able to stratify OS in the presence of venous invasion (GMS 0 vs GMS 2; HR 2.10 95% CI 1.04-4.26, *p*<0.04). Finally, in MMR-proficient tumours, GMS was once again only able to stratify OS for GMS 0 vs GMS 1 (HR 1.70 95% CI 1.24-2.33, *p*=0.001, Figure 4.2D), but was unable to stratify OS in MMR-deficient disease (*p*=0.61; Figure 4.2E).

Table 4.4. Overall survival for GMS according to mode of presentation, TNM stage, venous invasion, and MMR status in patients undergoing curative resection for stage I-III colon cancer (AP TMA).

Group (GMS category)		**Overall Survival**			
	N	5-year OS (%; SE)	Events (*N*=304)	HR (95% CI)	*P*
Whole cohort				*Trend*	*0.019*
0	147	76 (4)	77	1.0 (reference)	
1	277	58 (3)	182	1.46 (1.12-1.90)	**0.006**
2	71	56 (6)	45	1.43 (0.99-2.06)	0.058
Elective presentation				*Trend*	*0.057*
0	117	81 (4)	57	1.0 (reference)	
1	179	64 (4)	114	1.47 (1.07-2.03)	**0.017**
2	45	62 (7)	25	1.36 (0.85-2.18)	0.20
Emergency presentation				*Trend*	*0.81*
0	30	57 (9)	20	1.0 (reference)	
1	98	48 (5)	68	1.17 (0.71-1.93)	0.54
2	26	46 (10)	20	1.19 (0.64-2.21)	0.59
Stage I-II (N 0)				*Trend*	*0.50*
0	112	77 (4)	61	1.0 (reference)	
1	175	68 (4)	107	1.20 (0.87-1.64)	0.27
2	34	55 (9)	19	1.24 (0.74-2.07)	0.42
Stage III (N 1-2)				*Trend*	*0.03*
0	35	74 (7)	16	1.0 (reference)	
1	102	42 (5)	74	2.07 (1.20-3.55)	**0.009**
2	37	57 (8)	26	1.75 (0.94-3.27)	0.07
Venous invasion absent				*Trend*	*0.28*
0	116	76 (4)	65	1.0 (reference)	
1	189	62 (4)	122	1.28 (0.95-1.73)	0.11
2	36	63 (8)	23	1.19 (0.74-1.92)	0.47
Venous invasion present				*Trend*	*<0.04*
0	31	77 (8)	12	1.0 (reference)	
1	88	51 (5)	60	2.22 (1.19-4.13)	**0.012**
2	35	49 (8)	22	2.10 (1.04-4.26)	**0.040**
MMR-proficient				*Trend*	*0.005*
0	104	80 (4)	51	1.0 (reference)	
1	227	57 (3)	154	1.70 (1.24-2.33)	**0.001**
2	60	58 (6)	37	1.49 (0.98-2.28)	0.064
MMR-deficient				*Trend*	*0.61*
0	43	67 (7)	26	1.0 (reference)	
1	49	65 (7)	28	0.92 (0.54-1.57)	0.76
2	10	50 (16)	7	1.40 (0.61-3.25)	0.43

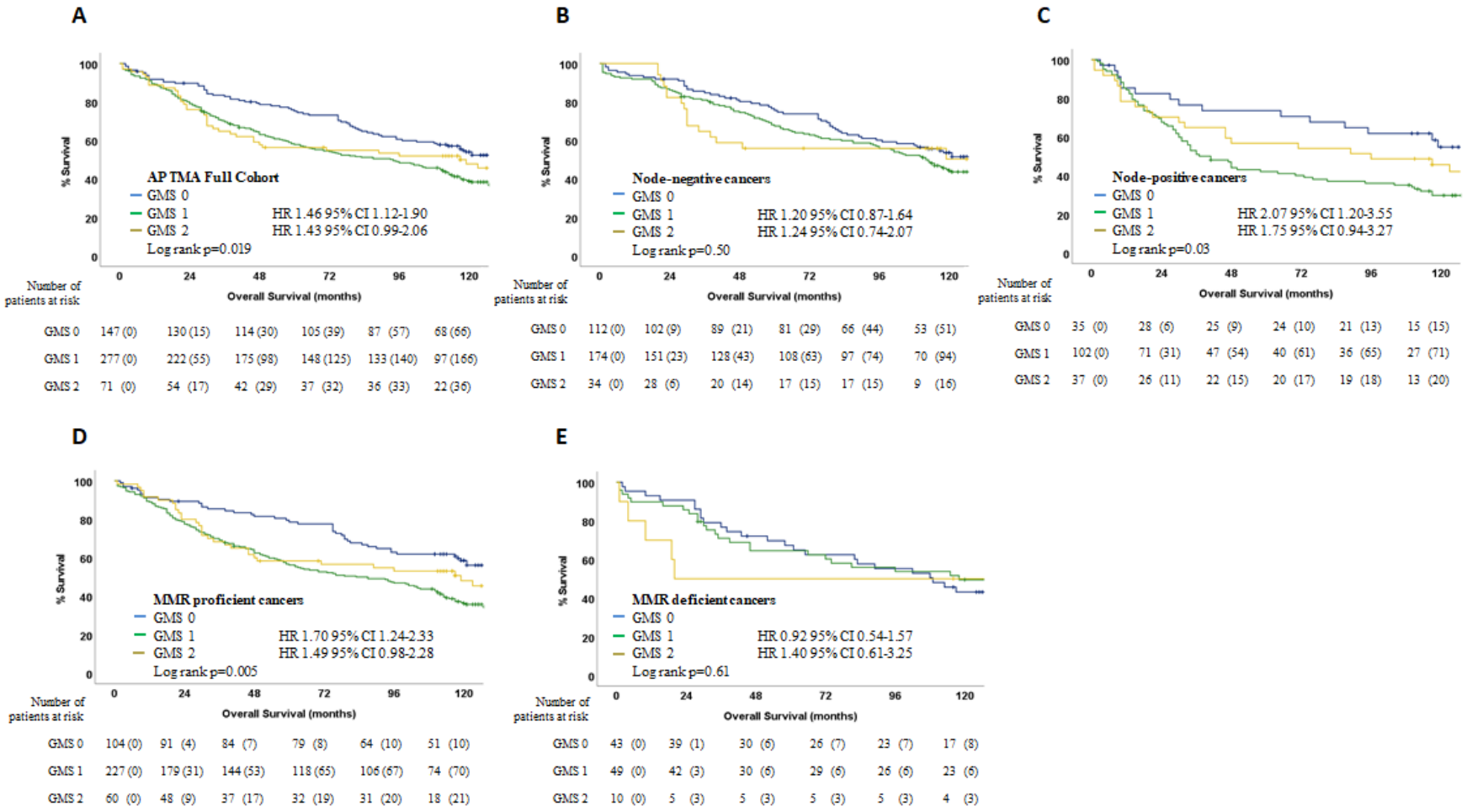


Figure 4.2. GMS stratification of OS according to nodal and MMR status in stage I-III colon cancer (AP TMA). (A) Full cohort (n=495), (B) Node-negative (n=320), (C) Node-positive (n=174), (D) MMR-proficient (n-391) and (E) MMR deficient (n=102).

Table 4.5. Overall survival analysis for clinicopathological characteristics in patients undergoing curative resection for stage I-III colon cancer (AP TMA).

Clinicopathological characteristics			**Overall survival**			
	N (%)[a]		Univariate HR (95% CI)	*P*	Multivariate HR (95% CI)	*P*
Age						
<65	125	(25)				
65-74	158	(32)				
>74	212	(43)	1.95 (1.66-2.28)	**<0.001**	2.13 (1.81-2.52)	**<0.001**
Gender						
Female	245	(49)				
Male	250	(51)	1.17 (0.93-1.46)	0.18		
Presentation						
Elective	341	(69)				
Emergency	154	(31)	1.60 (1.26-2.02)	**<0.001**	1.71 (1.34-2.17)	**<0.001**
TNM						
I-II	321	(65)				
III	174	(35)	1.31 (1.10-1.56)	**0.002**[b]		
T-stage						
1	21	(4)				
2	53	(11)				
3	279	(56)				
4	142	(29)	1.23 (1.06-1.44)	**0.008**	–	0.23
N-stage						
0	320	(65)				
1	117	(24)				
2	57	(12)	1.30 (1.11-1.52)	**0.001**	1.33 (1.13-1.56)	**<0.001**
Differentiation						
Well/mod	444	(90)				
Poor	51	(10)	1.46 (1.02-2.09)	**0.04**	–	0.08
Venous invasion						
Absent	341	(69)				
Present	154	(31)	1.15 (0.90-1.47)	0.26		
Tumour budding						
Present	123	(25)				
Absent	372	(75)	1.12 (0.87-1.45)	0.37		
MMR						
Proficient	391	(79)				
Deficient	102	(21)	0.94 (0.71-1.24)	0.65		
Tumour perforation						
No	464	(94)				
Yes	31	(6)	1.06 (0.84-1.33)	0.64		
Lymph node yield						
≥12	304	(61)				
<12	191	(39)	1.11 (0.88-1.40)	0.37		
Peritoneal involvement						
No	360	(73)				
Yes	135	(27)	1.27 (0.99-1.63)	0.06	–	0.93
GMS						
0	147	(30)				
1	277	(56)				
2	71	(14)	1.23 (1.04-1.46)	**0.017**	1.25 (1.05-1.50)	**0.015**

[a]percentages rounded to nearest whole number and may not total 100%

[b]not included in multivariate model as T-stage and N-stage included separately

4.2.2 Implications of Results in the AP TMA cohort

Whilst the original study using the JP TMA cohort looked at colorectal cancer as a whole, this study aimed to replicate those results in an entirely independent cohort (the AP TMA cohort was therefore chosen for this purpose), whilst also selecting out purely colon cancers. The reason for selecting only colon cancers was that there had been some anecdotal problems encountered in assessing TSP in rectal cancers following radiotherapy as this produced a fibrotic response(Hav et al., 2015). Therefore, in excluding the rectal cancers, the hope was that we would have a purer cohort to assess the effects of GMS. There were differences in the construction of the two patient cohorts. Whilst the JP TMA cohort was prospectively collected from consecutive colorectal cancers identified at a single hospital's multidisciplinary team meetings, the AP TMA cohort was a curated series identified retrospectively by selecting patients from a range of other hospitals, whose pathology samples were processed in the same laboratory. This may have had some impact on the results encountered.

GMS stratified cancer-specific survival as expected from the previous study(Park et al., 2015), with a good prognosis indicated by GMS 0 and a poor prognosis indicated by GMS 2, with GMS 1 having on the whole an intermediate outcome. This seemed to be in keeping with previous results. The exception, however, was in node-positive subgroup where a sharp decline was seen in CSS in the GMS 1 group on Kaplan-Meier analysis (Fig 5.1C). Similarly, when assessing overall survival, the GMS 1 group had a consistently poor outcome across a range of subgroups, worse on many occasions than that of GMS 2, which was unexpected as it did not fit with our hypothesis that GMS 2 was a phenotype with poor prognosis.

It is known that GMS 1 represents a heterogenous group with a likely wide range of genetic and transcriptomic tumour-related factors, whilst GMS 2 are categorised by high tumour stroma, which is generally a poor prognostic indicator.

It is possible that the curated nature of the series may have removed an element of normal distribution, thereby biasing the survival of the GMS 1 subgroup, or it could be the nature of some of the heterogenous tumours in GMS 1 that contributed to the observed survival differences. Furthermore, the decision to study GMS in a purely colonic setting, rather than colon and rectal may have been responsible for the different outcomes.

In order to investigate this further, a newly fashioned consecutive TMA cohort (the GRI-CRC-TMA) was selected to further assess these results in colon cancers.

4.3 VALIDATING THE GMS IN COLON CANCER IN THE GRI-CRC-TMA

4.3.1 Results of survival analysis in the GRI-CRC-TMA cohort

In the GRI-CRC-TMA cohort, there were 554 patients with stage I-III colon cancer and a valid GMS. Clinicopathological characteristics are presented in Table 4.6. Sixty-three percent of patients were younger than 75 at the time of surgery and 37% were node-positive. Ninety-three patients (17%) were GMS 0, 359 patients (65%) were GMS 1 and 102 patients (18%) were GMS 2. The median length of follow up of survivors was 10.4 years (Interquartile range: 8.4-14.3). During follow up there were 340 deaths, of which 139 were colon cancer-related. Five-year CSS was 80% across the cohort. For stages I, II and III, respectively, 5-year CSS rates were 95%, 87% and 66%.

Table 4.6. Clinicopathological characteristics and their relation to GMS in patients undergoing curative resection for colon cancer (GRI-CRC-TMA)

Clinicopathological characteristics			**GMS**						
	N (%)[a]		**0** (n=93) *N*	(%)	**1** (n=359) *N*	(%)	**2** (n=102) *N*	(%)	*P*
Age									
<65	166	(30)	36	(39)	102	(28)	28	(28)	0.41
65-74	181	(33)	23	(25)	120	(37)	38	(37)	
>74	207	(37)	34	(37)	137	(35)	36	(35)	
Gender									
Female	262	(47)	38	(41)	182	(51)	42	(41)	0.97
Male	292	(53)	55	(59)	177	(49)	60	(59)	
Presentation									
Elective	494	(89)	86	(93)	324	(90)	84	(82)	**0.02**
Emergency	60	(11)	7	(8)	35	(10)	18	(18)	
TNM									
I-II	349	(63)	75	(75)	232	(65)	42	(41)	**<0.001**
III	205	(27)	18	(19)	127	(35)	60	(59)	
T-stage									
1	23	(4)	7	(8)	16	(5)	0		**<0.001**
2	56	(10)	22	(24)	32	(9)	2	(2)	
3	305	(55)	51	(55)	202	(56)	52	(51)	
4	170	(31)	13	(14)	109	(30)	48	(47)	
N-stage									
0	349	(63)	75	(81)	232	(65)	42	(41)	**<0.001**
1	151	(27)	16	(17)	93	(26)	42	(41)	
2	54	(10)	2	(2)	34	(10)	18	(18)	
Differentiation									
Well/mod	491	(89)	90	(97)	310	(87)	91	(89)	0.11
Poor	60	(9)	3	(3)	46	(13)	11	(11)	
Venous invasion									
Absent	269	(49)	58	(62)	175	(49)	36	(35)	**<0.001**
Present	285	(51)	35	(38)	184	(51)	66	(65)	

[a]percentages rounded to nearest whole number – may not total 100%

Associations between GMS and CSS were assessed (Table 4.7). CSS was stratified by GMS in the whole cohort with 5-year CSS of 94%, 81% and 65% for GMS 0, 1 and 2, respectively (GMS 0 vs GMS 2: HR 3.54 95% CI 1.92-6.61, $p<0.001$; Figure 4.3A). On multivariate analysis for CSS, GMS remained independent (p=0.02) of age (p=0.002), emergency presentation (p=0.04), T-stage (p=0.001), N-stage (p<0.001), and tumours with <12 nodes sampled (p=0.01), (Table 4.8). Subgroup analysis was performed according to mode of presentation, node-negative/-positive disease, and venous invasion (Table 4.7). GMS stratified CSS for elective presentation (GMS 0 vs GMS 2; HR 3.21 95% CI 1.64-6.30, $p=0.002$), but was unable to stratify CSS in emergency presentation, due to small sample size. GMS stratified CSS for node-negative (GMS 0 vs GMS 2; HR 2.62 95% CI 1.13-6.07, $p<0.05$), (Figure 4.3B), as well as node-positive disease, although the overall trend was not significant (GMS 0 vs GMS 2; HR 3.14 95% CI 1.10-8.95, $p=0.06$), (Figure 4.3C). GMS stratified CSS regardless of the presence of venous invasion (venous invasion absent: GMS 0 vs GMS 2; HR 2.80 95% CI 1.24-6.31, $p=0.01$; venous invasion present: GMS 0 vs GMS 2; HR 4.79 95% CI 1.67-13.74, $p=0.007$).

Table 4.7. Cancer-specific survival for GMS according to mode of presentation, TNM stage, and venous invasion in patients undergoing curative resection for stage I-III colon cancer (GRI-CRC-TMA).

Group **GMS category**	**Survival**						
	N	5-year CSS (%; SE)	Events (*N*=139)		HR (95% CI)		*P*
Whole cohort					*Trend*		*<0.001*
0	93	94 (2)	14		1.0 (reference)		
1	359	81 (2)	85		1.78 (1.01-3.13)		**0.046**
2	102	65 (5)	40		3.54 (1.92-6.51)		**<0.001**
Elective presentation					*Trend*		*0.002*
0	86	95 (2)	12		1.0 (reference)		
1	324	82 (2)	73		1.83 (0.99-3.37)		0.05
2	84	69 (5)	29		3.21 (1.64-6.30)		**<0.001**
Emergency presentation					*Trend*		*<0.05*
0	7	86 (13)	2		1.0 (reference)		
1	35	70 (8)	12		1.39 (0.31-6.22)		0.31
2	18	63 (9)	11		3.62 (0.79-16.53)		0.11
Stage I-II (N 0)					*Trend*		*<0.05*
0	75	96 (2)	10		1.0 (reference)		
1	232	88 (2)	37		1.29 (0.64-2.59)		0.48
2	42	79 (7)	12		2.62 (1.13-6.07)		**0.03**
Stage III (N 1-2)					*Trend*		*0.06*
0	18	89 (8)	4		1.0 (reference)		
1	127	68 (4)	48		2.11 (0.76-5.85)		0.15
2	60	55 (7)	28		3.14 (1.10-8.95)		**0.03**
Venous invasion absent					*Trend*		*0.01*
0	58	94 (3)	10		1.0 (reference)		
1	175	86 (3)	33		1.17 (0.58-2.37)		0.67
2	36	64 (8)	14		2.80 (1.24-6.31)		**0.01**
Venous invasion present					*Trend*		*0.007*
0	35	94 (4)	4		1.0 (reference)		
1	184	75 (3)	52		2.92 (1.06-8.08)		**0.04**
2	66	66 (6)	26		4.79 (1.67-13.74)		**0.004**

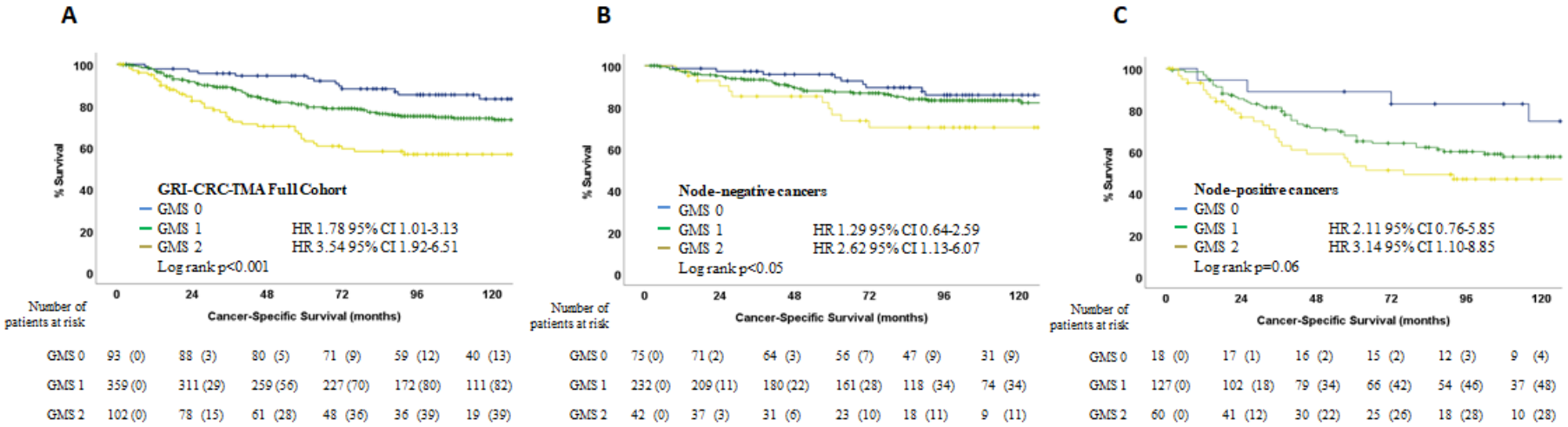


Figure 4.3. GMS stratification of CSS according to nodal status in stage I-III colon cancer (GRI-CRC-TMA). (A) Full cohort (n=554), (B) Node-negative (n=349), (C) Node-positive (n=205)

Table 4.8. Cancer-specific survival analysis for clinicopathological characteristics in patients undergoing curative resection for stage I-III colon cancer (GRI-CRC-TMA).

Clinicopathological characteristics			**Cancer-specific survival**			
	N (%)[a]		Univariate HR (95% CI)	*P*	Multivariate HR (95% CI)	*P*
Age						
<65	166	(30)				
65-74	181	(33)				
>74	207	(37)	1.37 (1.11-1.69)	**0.003**	1.41 (1.14-1.74)	**0.002**
Gender						
Female	262	(47)				
Male	292	(53)	1.01 (0.73-1.41)	0.94		
Presentation						
Elective	494	(89)				
Emergency	60	(11)	2.13 (1.38-3.28)	**<0.001**	1.62 (1.04-2.54)	**0.03**
TNM						
I-II	349	(63)				
III	205	(27)	2.46 (1.84-3.28)	**<0.001**[b]		
T-stage						
1	23	(4)				
2	56	(10)				
3	305	(55)				
4	170	(31)	2.00 (1.53-2.60)	**<0.001**	1.59 (1.20-2.10)	**0.001**
N-stage						
0	349	(63)				
1	151	(27)				
2	54	(10)	1.95 (1.57-2.42)	**<0.001**	1.73 (1.37-2.19)	**<0.001**
Differentiation						
Well/mod	491	(89)				
Poor	60	(9)	1.15 (0.67-1.96)	0.61		
Venous invasion						
Absent	269	(49)				
Present	285	(51)	1.52 (1.08-2.13)	**0.02**	–	0.67
Tumour perforation						
No	539	(97)				
Yes	15	(3)	1.35 (0.50-3.64)	0.56		
Lymph node yield						
≥12	412	(74)				
<12	142	(36)	1.42 (0.99-2.03)	0.06	1.60 (1.11-2.31)	**0.01**
Peritoneal involvement						
No	400	(72)				
Yes	154	(28)	2.35 (1.68-3.29)	**<0.001**	–	0.75
GMS						
0	93	(17)				
1	359	(65)				
2	102	(18)	1.92 (1.44-2.55)	**<0.001**	1.44 (1.07-1.94)	**0.02**

[a]percentages rounded to nearest whole number and may not total 100%

[b]not included in multivariate model as T-stage and N-stage included separately

Bold indicates $p<0.05$

Associations between GMS and OS were assessed (Table 4.9). OS was stratified by GMS in the whole cohort with 5-year OS of 84%, 67% and 53% for GMS 0, 1 and 2, respectively (GMS 0 vs GMS 2: HR 1.95 95% CI 1.34-2.83, *p*=0.002; Figure 4.4A). On multivariate analysis for OS, GMS was not independent of age (p<0.002), T-stage (p<0.001) or N-stage (p=0.002), Table 4.10. Subgroup analysis was performed according to mode of presentation, node-negative/-positive disease, and venous invasion (Table 4.9). GMS stratified OS for elective presentation (GMS 0 vs GMS 2; HR 1.89 95% CI 1.25-2.85, *p*=0.009), but was unable to stratify OS in emergency presentation, likely due to small sample size. GMS did not stratify OS for node-negative disease (*p*=0.20; Figure 4.4B) but did stratify OS in node-positive disease (GMS 0 vs GMS 2; HR 2.18 95% CI 1.09-4.36, *p*=0.027), (Figure 4.4C). GMS was unable to stratify OS in the absence of venous invasion (*p*=0.24) but did stratify OS when venous invasion was present (GMS 0 vs GMS 2; HR 2.43 95% CI 1.35-4.38, *p*=0.012).

Table 4.9. Overall survival for GMS according to mode of presentation, TNM stage and venous invasion in patients undergoing curative resection for stage I-III colon cancer (GRI-CRC-TMA).

Group **GMS category**		**Survival**				
	N	5-year OS (%; SE)	Events (*N*=340)		HR (95% CI)	*P*
Whole cohort					*Trend*	*0.002*
0	93	84 (4)	47		1.0 (reference)	
1	359	67 (2)	225		1.40 (1.03-1.92)	**0.035**
2	102	53 (5)	68		1.95 (1.34-2.83)	**<0.001**
Elective presentation					*Trend*	*0.009*
0	86	84 (4)	40		1.0 (reference)	
1	324	68 (3)	202		1.55 (1.10-2.17)	**0.012**
2	84	58 (5)	53		1.89 (1.25-2.85)	**0.003**
Emergency presentation					*Trend*	*0.028*
0	7	86 (13)	7		1.0 (reference)	
1	35	63 (8)	23		0.61 (0.26-1.44)	0.26
2	18	28 (11)	15		1.56 (0.63-3.89)	0.63
Stage I-II (N 0)					*Trend*	*0.20*
0	75	84 (4)	37		1.0 (reference)	
1	232	73 (3)	138		1.28 (0.89-1.84)	0.19
2	42	64 (7)	25		1.58 (0.95-2.62)	0.08
Stage III (N 1-2)					*Trend*	*0.06*
0	18	83 (9)	10		1.0 (reference)	
1	127	57 (4)	87		1.61 (0.84-3.12)	0.15
2	60	45 (6)	43		2.18 (1.09-4.36)	**0.027**
Venous invasion absent					*Trend*	*0.24*
0	58	84 (5)	32		1.0 (reference)	
1	175	72 (3)	112		1.17 (0.81-1.78)	0.38
2	36	53 (8)	25		2.80 (0.93-2.65)	0.09
Venous invasion present					*Trend*	*0.012*
0	35	83 (6)	15		1.0 (reference)	
1	184	63 (4)	113		1.77 (1.03-3.04)	**0.038**
2	66	53 (6)	43		2.43 (1.35-4.38)	**0.003**

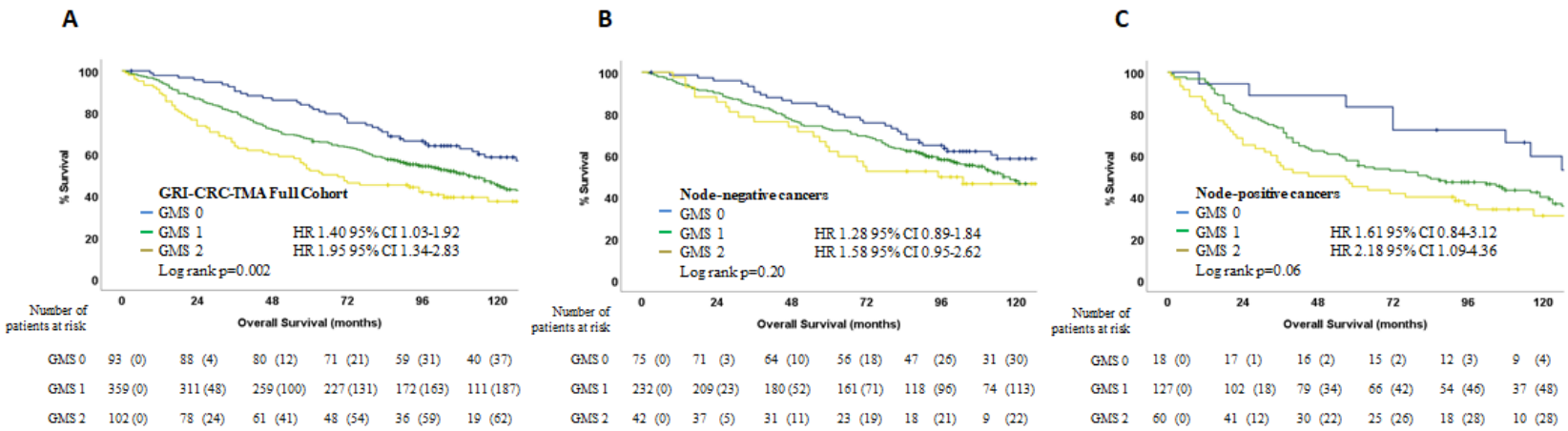


Figure 4.4. GMS stratification of OS according to nodal status in stage I-III colon cancer (GRI-CRC-TMA). (A) Full cohort (n=554), (B) Node-negative (n=349), (C) Node-positive (n=205)

Table 4.10. Overall survival analysis for clinicopathological characteristics in patients undergoing curative resection for stage I-III colon cancer (GRI-CRC-TMA).

Clinicopathological characteristics			**Overall survival**			
	N (%)[a]		Univariate HR (95% CI)	*P*	Multivariate HR (95% CI)	*P*
Age						
<65	166	(30)				
65-74	181	(33)				
>74	207	(37)	1.78 (1.62-2.15)	**<0.001**	1.92 (1.66-2.21)	**<0.001**
Gender						
Female	262	(47)				
Male	292	(53)	1.04 (0.84-1.28)	0.75		
Presentation						
Elective	494	(89)				
Emergency	60	(11)	1.45 (1.06-1.99)	**0.02**	–	0.35
TNM						
I-II	349	(63)				
III	205	(27)	1.38 (1.17-1.63)	**<0.001**[b]		
T-stage						
1	23	(4)				
2	56	(10)				
3	305	(55)				
4	170	(31)	1.35 (1.16-1.58)	**<0.001**	1.34 (1.14-1.57)	**<0.001**
N-stage						
0	349	(63)				
1	151	(27)				
2	54	(10)	1.32 (1.13-1.53)	**<0.001**	1.29 (1.10-1.51)	**0.002**
Differentiation						
Well/mod	491	(89)				
Poor	60	(9)	1.29 (0.92-1.80)	0.14		
Venous invasion						
Absent	269	(49)				
Present	285	(51)	1.19 (0.96-1.47)	0.12		
Tumour perforation						
No	539	(97)				
Yes	15	(3)	1.72 (0.97-3.07)	0.06		
Lymph node yield						
≥12	412	(74)				
<12	142	(36)	1.13 (0.89-1.44)	0.31		
Peritoneal involvement						
No	400	(72)				
Yes	154	(28)	1.40 (1.12-1.76)	**0.004**	–	0.88
GMS						
0	93	(17)				
1	359	(65)				
2	102	(18)	1.39 (1.16-1.68)	<**0.001**	–	0.10

[a]percentages rounded to nearest whole number and may not total 100%

[b]not included in multivariate model as T-stage and N-stage included separately

4.3.2 Implications of Results in the GRI-CRC-TMA cohort

This further independent cohort assessed colon cancers and survival functions with regard to GMS and the relationship between GMS and other clinicopathological characteristics. Colon cancers alone were assessed in this cohort as explained above with regard to anecdotal problems encountered in assessing TSP in rectal cancers following radiotherapy as this produced a fibrotic response(Hav et al., 2015). Therefore, in excluding the rectal cancers, the hope was that we would have a purer cohort to assess the effects of GMS. In addition, given the unexplained reversal of the GMS 1 and 2 curves on the Kaplan Meier charts for GMS in the AP TMA cohort, it was unclear whether the curated nature of the AP TMA may have a played a role in the results of section 4.2.1. Therefore, the GMS was assessed in a prospectively collected cohort of colon cancers: the GRI-CRC-TMA cohort.

GMS stratified cancer-specific survival as expected from the original study findings(Park et al., 2015), with a good prognosis indicated by GMS 0 and a poor prognosis indicated by GMS 2, with GMS 1 having an intermediate outcome. The three survival bands stratified CSS and OS as in the original study with no reversal of GMS 1 and 2 evident in this cohort.

4.3.3 Implications of Results from both the AP TMA and GRI-CRC-TMA cohorts

When the results for the two cohorts are analysed together, the first difference to note is that the AP TMA cohort is slightly older, with 43% of patients aged 75 or older vs 37% in this category for the GRI-CRC-TMA. Furthermore, there were significantly more emergency presentations in the AP TMA cohort with 31% presenting as an emergency vs 11% in the GRI-CRC-TMA. The increase in emergency presentations was not limited to one GMS category. In terms of GMS category, in the AP TMA the rates of emergency presentation were 20%, 35% and 37% vs 8%, 10% and 18% in the GRI-CRC-TMA for GMS 0, 1 and 2, respectively. Therefore, the rise in emergency presentations was

disproportionately higher in GMS 1 in the AP TMA. There were also much higher levels of venous invasion in the GRI-CRC-TMA cohort with 51% vs 31% in the AP TMA. However, this latter difference may be due to the greater historical use of elastin in the assessment of venous invasion in the Glasgow Royal Infirmary laboratories. Having noted these differences, there were similar proportions of males to females, TNM stage, T-stage, N-stage and tumour differentiation between the two cohorts.

In terms of GMS assessment, there was similar stratification of both cohorts into three survival bands for both cohorts, except with regard to the unexpected reversal of GMS 1 and GMS 2 in the AP TMA. When this is viewed in retrospect, the elevated rate of emergency presentations in the AP TMA, which was noted to be significant for both CSS and OS in both cohorts may explain the difference in the behaviour of the GMS 1 curve in the AP TMA cohort.

4.4 VALIDATING THE GMS IN THE COMBINED JP-AP TMA COHORT

The data for the following cohort were published in the British Journal of Cancer(Alexander et al., 2020c). In order to expand the numbers available for analysis, the colorectal cancers from the combined JP-AP TMA cohort were assessed. These included, therefore, some of the patients from the original study. However, since a further independent cohort was to be used in the GMS validation (the TransScot cohort), the use of the expanded cohort was deemed acceptable since it allowed observations to be made comparing not only survival data, but also a limited analysis of genetic data and immunohistochemical data for T-cell subsets.

4.4.1 Results of survival analysis in the combined JP-AP TMA cohort

In the combined JP-AP TMA cohort, there were 862 patients with TNM I-III CRC. Clinicopathological characteristics are presented in Table 4.11. Sixty percent of patients were younger than 75 years at time of surgery, and 35% were node-positive. Fifty-eight

percent had low-risk disease (according to the Petersen Index; low-risk: TNM I-II and Petersen Index <2; high-risk: TNM II and Petersen index ≥2 or TNM III), while 42% had high-risk disease. Of the high-risk group, 61 were high-risk TNM II, whereas 302 were TNM III. Three hundred (35%) patients were GMS 0, 424 (49%) patients GMS 1 and 138 (16%) patients GMS 2. Median follow-up for all patients was 7.96 years (range: 2.3-11.1). There were 554 deaths and 271 patients developed recurrence.

Associations between GMS and DFS were assessed (Table 4.11). GMS stratified survival in the whole cohort for DFS with 5-year DFS for GMS 0, 1 and 2 of 71%, 58% and 46%, respectively (GMS 0 vs GMS 2: HR 1.50 95% CI 1.16-1.93, p=0.002; Figure 4.5A). On multivariate analysis for DFS, GMS remained independent (p=0.004) of age (p<0.001), T-stage (p=0.003), N-stage (p<0.001) and mGPS (p<0.001). Subgroup analysis was performed according to clinical risk (according to the Petersen index) and primary tumour site (Table 4.12). While GMS did not stratify survival in low-risk disease (Figure 4.5B), high-risk disease was stratified with 5-year DFS for GMS 0, 1 and 2 of 66%, 43% and 38%, respectively (GMS 0 vs GMS 2: HR 1.72 95% CI 1.19-2.47, p=0.003; Figure 4.5C). In addition, GMS was able to stratify 5-year DFS for colon cancer with GMS 0, 1 and 2 of 72%, 58% and 45%, respectively (GMS 0 vs GMS 2: HR 1.57 95% CI 1.16-2.12, p=0.004; Figure 4.6A), but not rectal cancer (Figure 4.6B).

Table 4.11. Disease-free and relapse-free survival in stage I-III colorectal cancer and associations of clinicopathological features with GMS in patients in the JP-AP TMA (*N*=862).

Clinicopathological characteristics		**Disease-free survival**				**Relapse-free survival**				**GMS category**						
	N (%)[a]	Univariate HR (95% CI)	*P*	Multivariate HR (95% CI)	*P*	Univariate HR (95% CI)	*P*	Multivariate HR (95% CI)	*P*	**0** (n=300) *N* (%)[a]		**1** (n=424) *N* (%)		**2** (n=138) *N* (%)		*Pears on* X^2
Age																
≤64	245 (28)									80	(27)	111	(26)	54	(39)	**0.04**
65-74	276 (32)									98	(33)	138	(33)	40	(29)	
≥75	341 (40)	1.66 (1.48-1.86)	**<0.001**	1.71 (1.51-1.95)	**<0.001**	1.10 (0.95-1.28)	0.20	–	–	122	(41)	175	(41)	44	(32)	
Gender																
Female	419 (49)									151	(50)	207	(49)	61	(44)	0.27
Male	443 (51)	1.12 (0.95-1.33)	0.18	–	–	1.15 (0.91-1.47)	0.24	–	–	149	(50)	217	(51)	77	(56)	
Presentation																
Elective	686 (80)									260	(87)	319	(75)	107	(78)	**0.002**
Emergency	175 (20)	1.55 (1.27-1.89)	**<0.001**	–	0.17	1.94 (1.49-2.53)	**<0.001**	–	0.10	39	(13)	105	(25)	31	(22)	
TNM																
I-II (low-risk)	499 (58)									201	(67)	244	(58)	54	(39)	**<0.001**
II-III (high-risk)	363 (42)	1.58 (1.33-1.87)	**<0.001**[b]	–	–	3.02 (2.36-3.87)	**<0.001**[b]	–	–	99	(33)	180	(42)	84	(61)	
T-stage																
T1	42 (5)									28	(9)	13	(3)	1	(1)	**<0.001**
T2	113 (13)									65	(22)	39	(9)	9	(7)	
T3	488 (57)									150	(50)	260	(61)	78	(56)	
T4a	179 (20)									43	(14)	93	(22)	43	(31)	
T4b	40 (5)	1.30 (1.15-1.46)	**<0.001**	1.25 (1.08-1.44)	**0.003**	1.81 (1.51-2.16)	**<0.001**	1.46 (1.17-1.82)	**0.001**	14	(5)	19	(5)	7	(6)	

N-stage																
N0	556 (65)									217	(73)	272	(64)	67	(49)	**<0.001**
N1	218 (25)									66	(22)	105	(25)	47	(34)	
N2	84 (10)	1.38 (1.22-1.55)	**<0.001**	1.31 (1.14-1.51)	**<0.001**	1.96 (1.68-2.30)	**<0.001**	1.58 (1.32-1.90)	**<0.001**	16	(5)	45	(11)	23	(17)	
Site																
Colon	650 (75)									206	(69)	345	(81)	99	(72)	0.08
Rectum	212 (25)	0.93 (0.76-1.13)	0.44	–	–	0.96 (0.73-1.27)	0.78			94	(31)	79	(19)	39	(28)	
Differentiation																
Well/mod	775 (90)									271	(90)	379	(89)	125	(91)	0.95
Poor	87 (10)	1.36 (1.04-1.79)	**0.03**	–	0.46	1.61 (1.13-2.30)	**0.01**	–	0.87	29	(10)	45	(11)	13	(9)	
Venous invasion																
Absent	589 (68)									226	(75)	290	(69)	73	(53)	**<0.001**
Present	273 (32)	1.38 (1.15-1.65)	**<0.001**	–	0.18	1.86 (1.46-2.37)	**<0.001**	1.34 (1.01-1.76)	**0.04**	74	(25)	134	(31)	65	(47)	
Tumour budding																
Absent	618 (72)									219	(73)	304	(72)	95	(69)	0.39
Present	244 (28)	1.04 (0.86-1.25)	0.70	–	–	1.33 (1.03-1.71)	**0.03**	–	0.15	81	(27)	120	(28)	43	(31)	
KRAS status (*n*=212)																
Wild-type	111 (52)									36	(55)	53	(50)	22	(55)	0.86
Mutant	101 (48)	1.16 (0.84-1.59)	0.37	–	–	1.08 (0.72-1.61)	0.71	–	–	29	(45)	54	(50)	18	(45)	
BRAF status (*n*=212)																
Wild-type	182 (86)									54	(83)	91	(85)	37	(93)	0.21
Mutant	30 (14)	1.03 (0.66-1.59)	0.90	–	–	0.91 (0.51-1.64)	0.76	–	–	11	(17)	16	(15)	3	(7)	

MMR																
Proficient	686 (82)									230	(78)	342	(83)	114	(84)	0.10
Deficient	155 (18)	0.99 (0.80-1.23)	0.94	–	–	0.80 (0.57-1.11)	0.18	–	–	64	(22)	69	(17)	22	(16)	
mGPS																
0	386 (55)									152	(59)	175	(52)	59	(54)	0.19
1	201 (29)									68	(27)	102	(30)	31	(28)	
2	115 (16)	1.59 (1.40-1.79)	**<0.001**	1.52 (1.34-1.73)	**<0.001**	1.69 (1.43-1.99)	**<0.001**	1.59 (1.34-1.90)	**<0.001**	37	(14)	59	(18)	19	(17)	
GMS																
0	300 (35)									–		–		–		–
1	424 (49)									–		–		–		
2	138 (16)	1.24 (1.09-1.40)	**0.001**	1.24 (1.07-1.43)	**0.004**	1.76 (1.48-2.08)	**<0.001**	1.53 (1.26-1.86)	**<0.001**	–		–		–		

[a]percentages rounded to nearest whole number and may not total 100% [b]not included in multivariate model as T-stage and N-stage included separately

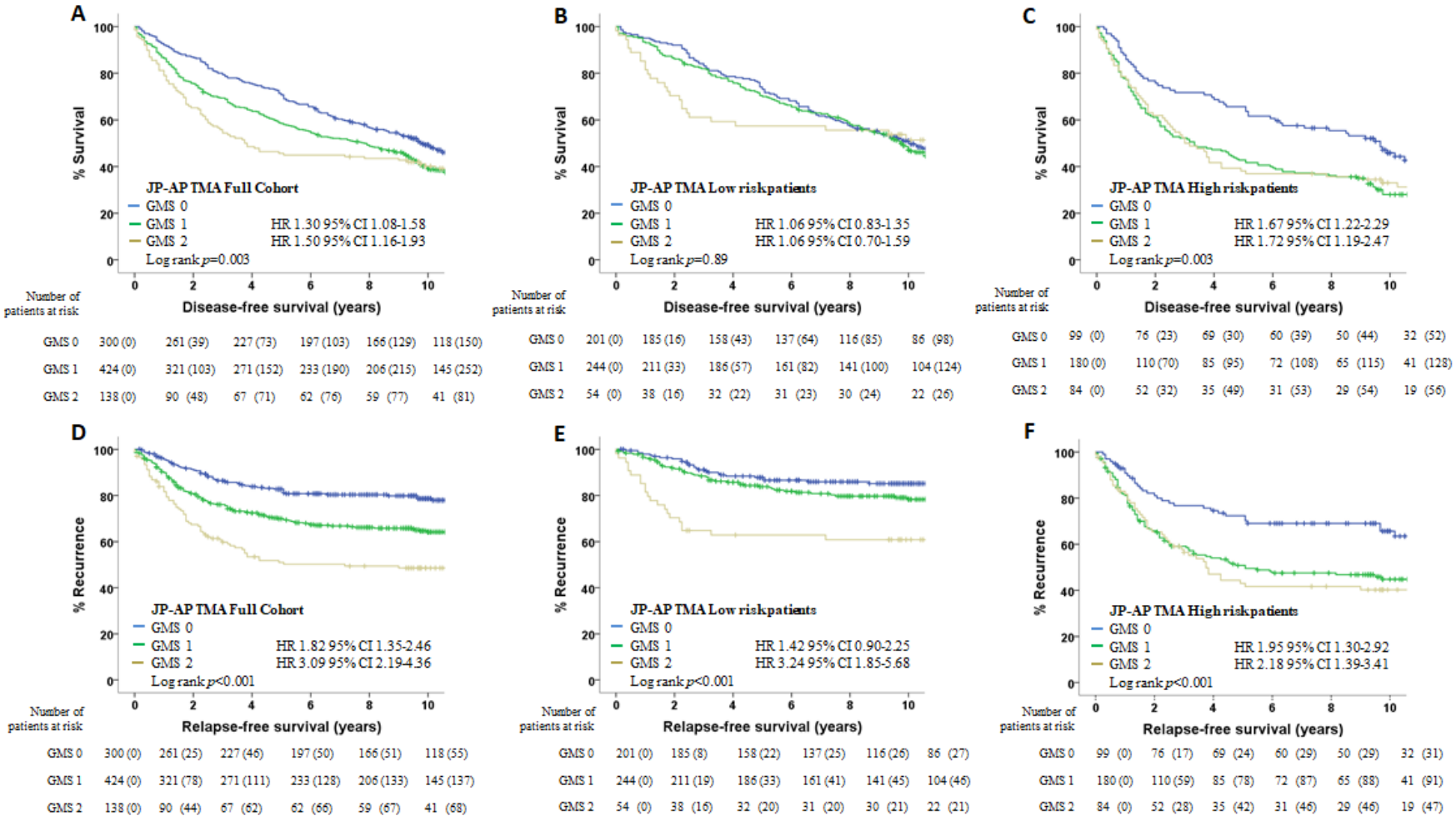


Figure 4.5. GMS and survival according to disease risk in JP-AP TMA cohort. (A-C) GMS and DFS in (A) full cohort (n=862), (B) "low-risk" CRC (n=499) and (C) "high-risk" CRC (n=363). (D-F) GMS and RFS in (D) full cohort (n=862), (E) "low-risk" CRC (n=499) and (F) "high-risk" CRC (n=363).

Table 4.12. Survival for GMS according to low- and high- risk disease and location of primary tumour in the JP-AP TMA (*N*=862).

Group **GMS category**		**Disease-free Survival**				**Relapse-free Survival**			
	N	5-year DFS (%; SE)	Events (*N*=541)	HR (95% CI)	*P*	5-year RFS (%; SE)	Events (*N*=271)	HR (95% CI)	*P*
Full cohort				*Trend*	*0.003*			*Trend*	*<0.001*
0	300	71 (3)	168	1.0 (reference)		83 (2)	61	1.0 (reference)	
1	424	58 (2)	281	1.30 (1.08-1.58)	**0.007**	70 (2)	141	1.82 (1.35-2.46)	**<0.001**
2	138	46 (4)	92	1.50 (1.16-1.93)	**0.002**	51 (4)	69	3.09 (2.19-4.36)	**<0.001**
TNM I-II (low-risk)				*Trend*	*0.89*			*Trend*	*<0.001*
0	201	73 (3)	113	1.0 (reference)		88 (2)	29	1.0 (reference)	
1	244	70 (3)	148	1.06 (0.83-1.35)	0.65	84 (2)	49	1.42 (0.90-2.25)	0.13
2	54	57 (7)	29	1.06 (0.70-1.59)	0.79	63 (7)	21	3.24 (1.85-5.68)	**<0.001**
TNM II-III (high-risk)				*Trend*	*0.003*			*Trend*	*0.001*
0	99	66 (5)	55	1.0 (reference)		72 (5)	32	1.0 (reference)	
1	180	43 (4)	133	1.67 (1.22-2.29)	**0.001**	51 (4)	92	1.95 (1.30-2.92)	**0.001**
2	84	38 (5)	63	1.72 (1.19-2.47)	**0.003**	43 (6)	48	2.18 (1.39-3.41)	**0.001**
Colon cancer				*Trend*	*0.004*			*Trend*	*<0.001*
0	206	72 (3)	113	1.0 (reference)		84 (3)	41	1.0 (reference)	
1	345	58 (3)	233	1.38 (1.10-1.73)	**0.005**	69 (3)	115	1.88 (1.32-2.68)	**0.001**
2	99	45 (5)	67	1.57 (1.16-2.12)	**0.004**	51 (5)	49	3.15 (2.08-4.77)	**<0.001**
Rectal Cancer				*Trend*	*0.46*			*Trend*	*0.003*
0	94	68 (5)	55	1.0 (reference)		80 (4)	20	1.0 (reference)	
1	79	62 (5)	48	1.07 (0.73-1.58)	0.72	72 (5)	26	1.66 (0.92-2.97)	0.09
2	39	46 (8)	25	1.35 (0.84-2.17)	0.21	51 (8)	20	2.95 (1.58-5.48)	**0.001**

Abbreviation: DFS, Disease-free survival; RFS, Relapse-free survival

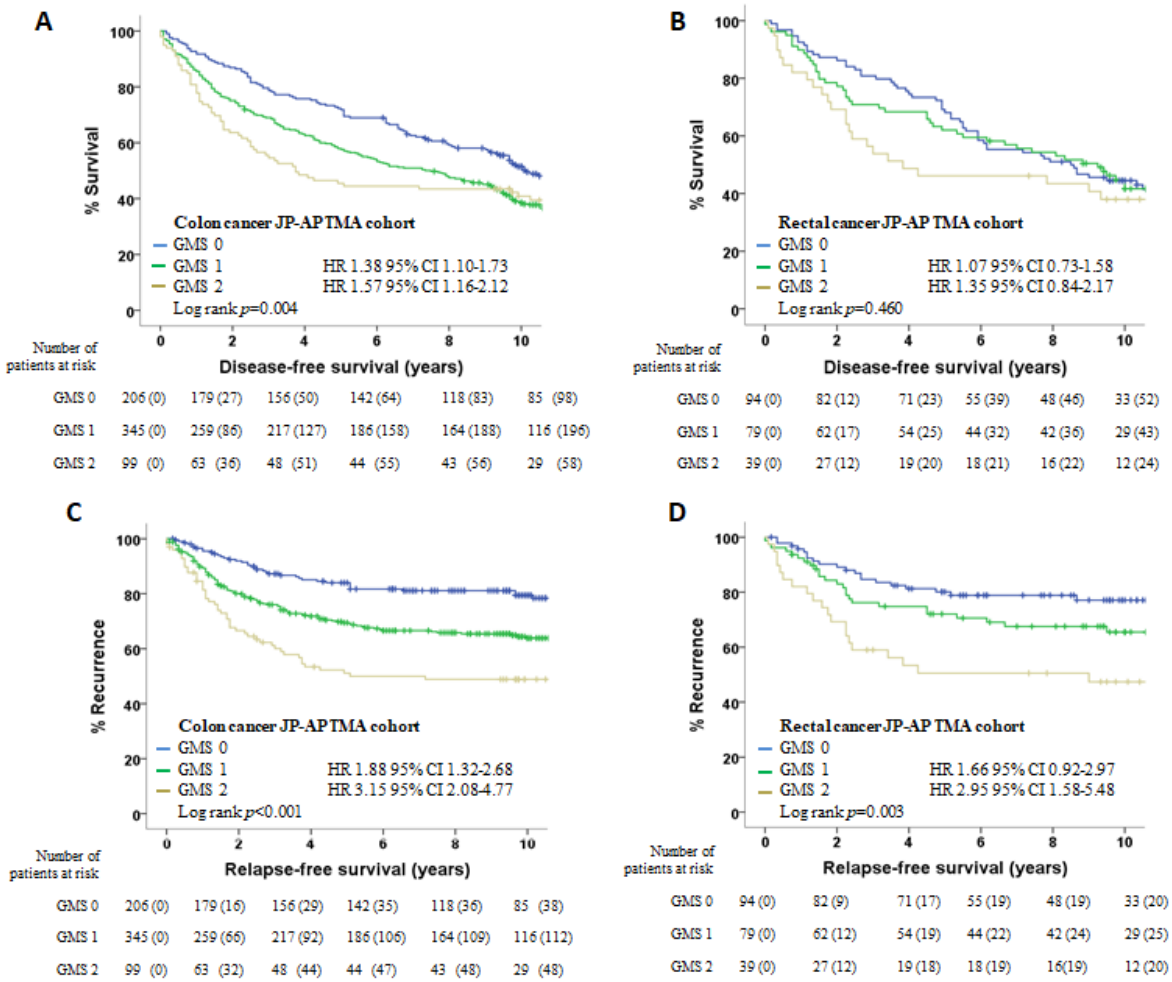


Figure 4.6. GMS and survival in colon and rectal cancer in JP-AP TMA cohort. (A, B) GMS and DFS in (A) colon (n=650) or (B) rectal cancer (n=212). (C, D) GMS and RFS in (C) colon (n=650) or (D) rectal cancer (n=212).

Next, associations between GMS and RFS were assessed (Table 4.11). GMS significantly stratified RFS for the whole cohort with 5-year RFS of 83%, 70% and 51% for GMS 0, 1 and 2, respectively (GMS 0 vs GMS 2: HR 3.09 95% CI 2.19-4.36, $p<0.001$, Figure 4.5D). On multivariate analysis for RFS, GMS remained associated with survival ($p<0.001$) independent of T-stage ($p=0.001$), N-stage ($p<0.001$), venous invasion ($p=0.04$) and mGPS ($p<0.001$). In low-risk disease (Table 4.12), 5-year RFS was 88%, 84% and 63% for GMS 0, 1 and 2, respectively, with GMS 2 associated with significantly worse RFS (GMS 0 vs GMS 2: HR 3.24 95% CI 1.85-5.68, $p<0.001$, Figure 4.5E). In high-risk disease (Table 4.12), 5-year RFS was 72%, 51% and 43% for GMS 0, 1 and 2, respectively, and GMS 0 had significantly better RFS (GMS 0 vs GMS 2: HR 2.18 95% CI 1.39-3.41, $p=0.001$, Figure 4.5F). On subgroup analysis by disease site (Table 4.12), GMS stratified RFS in patients with colon cancer ($n=650$), with 5-year RFS for GMS 0, 1 and 2 of 84%, 69% and 51%, respectively (GMS 0 vs GMS 2: HR 3.15 95% CI 2.08-4.77, $p<0.001$, Figure 4.6C), and rectal cancer ($n=212$), with 5-year RFS for GMS 0, 1 and 2 of 80%, 72% and 51%, respectively (GMS 0 vs GMS 2: HR 2.95 95% CI 1.58-5.48, $p=0.001$ Figure 4.6D).

Table 4.13. Overall and cancer-specific survival in stage I-III colorectal cancer in patients in the JP-AP TMA (*N*=862).

Clinicopathological characteristics		**Overall survival**				**Cancer-specific survival**			
	N (%)[a]	Univariate HR (95% CI)	*P*	Multivariate HR (95% CI)	*P*	Univariate HR (95% CI)	*P*	Multivariate HR (95% CI)	*P*
Age									
≤64	245 (28)								
65-74	276 (32)								
≥75	341 (40)	1.78 (1.59-2.00)	**<0.001**	1.88 (1.65-2.13)	**<0.001**	1.11 (0.95-1.30)	0.20	–	–
Gender									
Female	419 (49)								
Male	443 (51)	1.18 (0.99-1.39)	0.06	1.30 (1.07-1.58)	**0.007**	1.23 (0.95-1.60)	0.11	–	–
Presentation									
Elective	686 (80)								
Emergency	175 (20)	1.48 (1.21-1.82)	**<0.001**	–	0.23	1.87 (1.40-2.49)	**<0.001**	–	0.28
TNM									
I-II (low-risk)	499 (58)								
II-III (high-risk)	363 (42)	1.49 (1.26-1.76)	**<0.001**[b]	–	–	3.22 (2.46-4.22)	**<0.001**[b]	–	–
T-stage									
T1	42 (5)								
T2	113 (13)								
T3	488 (57)								
T4a	179 (20)								
T4b	40 (5)	1.23 (1.10-1.38)	**<0.001**	1.19 (1.03-1.36)	**0.02**	1.85 (1.52-2.25)	**<0.001**	1.43 (1.13-1.81)	**0.002**
N-stage									
N0	556 (65)								
N1	218 (25)								
N2	84 (10)	1.31 (1.17-1.48)	**<0.001**	1.29 (1.12-1.48)	<**0.001**	1.99 (1.69-2.34)	**<0.001**	1.55 (1.29-1.87)	<**0.001**
Site									
Colon	650 (75)								
Rectum	212 (25)	0.91 (0.75-1.11)	0.35	–	–	1.04 (0.78-1.39)	0.80		

Differentiation									
Well/mod	775 (90)								
Poor	87 (10)	1.32 (1.01-1.73)	0.05	–	0.40	1.69 (1.16-2.47)	**0.01**	–	0.63
Venous invasion									
Absent	589 (68)								
Present	273 (32)	1.38 (1.16-1.65)	**<0.001**	–	0.18	2.06 (1.60-2.67)	**<0.001**	1.48 (1.11-1.97)	**0.008**
Tumour budding									
Absent	618 (72)								
Present	244 (28)	1.04 (0.87-1.25)	0.67	–	–	1.33 (1.01-1.74)	**0.04**	–	0.16
KRAS status (*n*=212)									
Wild-type	111 (52)								
Mutant	101 (48)	1.21 (0.87-1.67)	0.26	–	–	1.11 (0.73-1.71)	0.62	–	–
BRAF status (*n*=212)									
Wild-type	182 (86)								
Mutant	30 (14)	0.85 (0.53-1.37)	0.51	–	–	0.69 (0.35-1.38)	0.30	–	–
MMR									
Proficient	686 (82)								
Deficient	155 (18)	1.03 (0.83-1.28)	0.82	–	–	0.88 (0.62-1.25)	0.46	–	–
mGPS									
0	386 (55)								
1	201 (29)								
2	115 (16)	1.58 (1.40-1.78)	**<0.001**	1.51 (1.33-1.71)	**<0.001**	1.75 (1.47-2.08)	**<0.001**	1.61 (1.35-1.93)	**<0.001**
GMS									
0	300 (35)								
1	424 (49)								
2	138 (16)	1.23 (1.09-1.39)	**0.001**	1.21 (1.05-1.39)	**0.009**	1.88 (1.56-2.25)	**<0.001**	1.63 (1.32-2.00)	**<0.001**

[a]percentages rounded to nearest whole number and may not total 100% [b]not included in multivariate model as T-stage and N-stage included separately

Table 4.14. Overall and Cancer-Specific Survival for GMS according to low- and high- risk disease and location of cancer in the JP-AP TMA

Group **GMS category**		**Overall Survival**				**Cancer-specific Survival**			
	N	5-year OS (%; SE)	Events (*N*=554)	HR (95% CI)	*P*	5-year CSS (%; SE)	Events (*N*=235)	HR (95% CI)	*P*
Full cohort				*Trend*	*0.003*			*Trend*	*<0.001*
0	300	69 (3)	176	1.0 (reference)		87 (2)	48	1.0 (reference)	
1	424	58 (2)	282	1.27 (1.05-1.53)	**0.015**	72 (2)	123	2.00 (1.43-2.79)	**<0.001**
2	138	48 (4)	96	1.50 (1.17-1.93)	**0.001**	55 (4)	64	3.55 (2.44-5.16)	**<0.001**
TNM I-II (low-risk)				*Trend*	*0.79*			*Trend*	*<0.001*
0	201	71 (3)	115	1.0 (reference)		93 (2)	20	1.0 (reference)	
1	244	68 (3)	152	1.08 (0.85-1.38)	0.52	86 (2)	42	1.74 (1.02-2.97)	**0.04**
2	54	61 (7)	31	1.09 (0.73-1.63)	0.66	66 (7)	19	3.94 (2.10-7.39)	**<0.001**
TNM II-III (high-risk)				*Trend*	*0.008*			*Trend*	*0.001*
0	99	64 (5)	61	1.0 (reference)		74 (5)	28	1.0 (reference)	
1	180	43 (4)	130	1.50 (1.10-2.03)	**0.01**	54 (4)	81	1.91 (1.24-2.93)	**0.003**
2	84	40 (5)	65	1.67 (1.18-2.38)	**0.004**	47 (6)	45	2.34 (1.46-3.76)	**<0.001**
Colon cancer				*Trend*	*0.02*			*Trend*	*<0.001*
0	206	72 (3)	120	1.0 (reference)		87 (2)	33	1.0 (reference)	
1	345	57 (3)	232	1.29 (1.04-1.61)	**0.02**	72 (3)	97	1.94 (1.30-2.87)	**0.001**
2	99	49 (5)	70	1.49 (1.11-2.00)	**0.008**	57 (5)	44	3.36 (2.14-5.27)	**<0.001**
Rectal Cancer				*Trend*	*0.21*			*Trend*	*<0.001*
0	94	62 (5)	56	1.0 (reference)		86 (4)	15	1.0 (reference)	
1	79	59 (6)	50	1.13 (0.77-1.65)	0.54	71 (5)	26	2.23 (1.18-4.20)	**0.014**
2	39	46 (8)	26	1.51 (0.95-2.41)	0.08	50 (8)	20	4.07 (2.08-7.96)	**0.001**

Abbreviation: OS, Overall survival; CSS, Cancer-specific survival

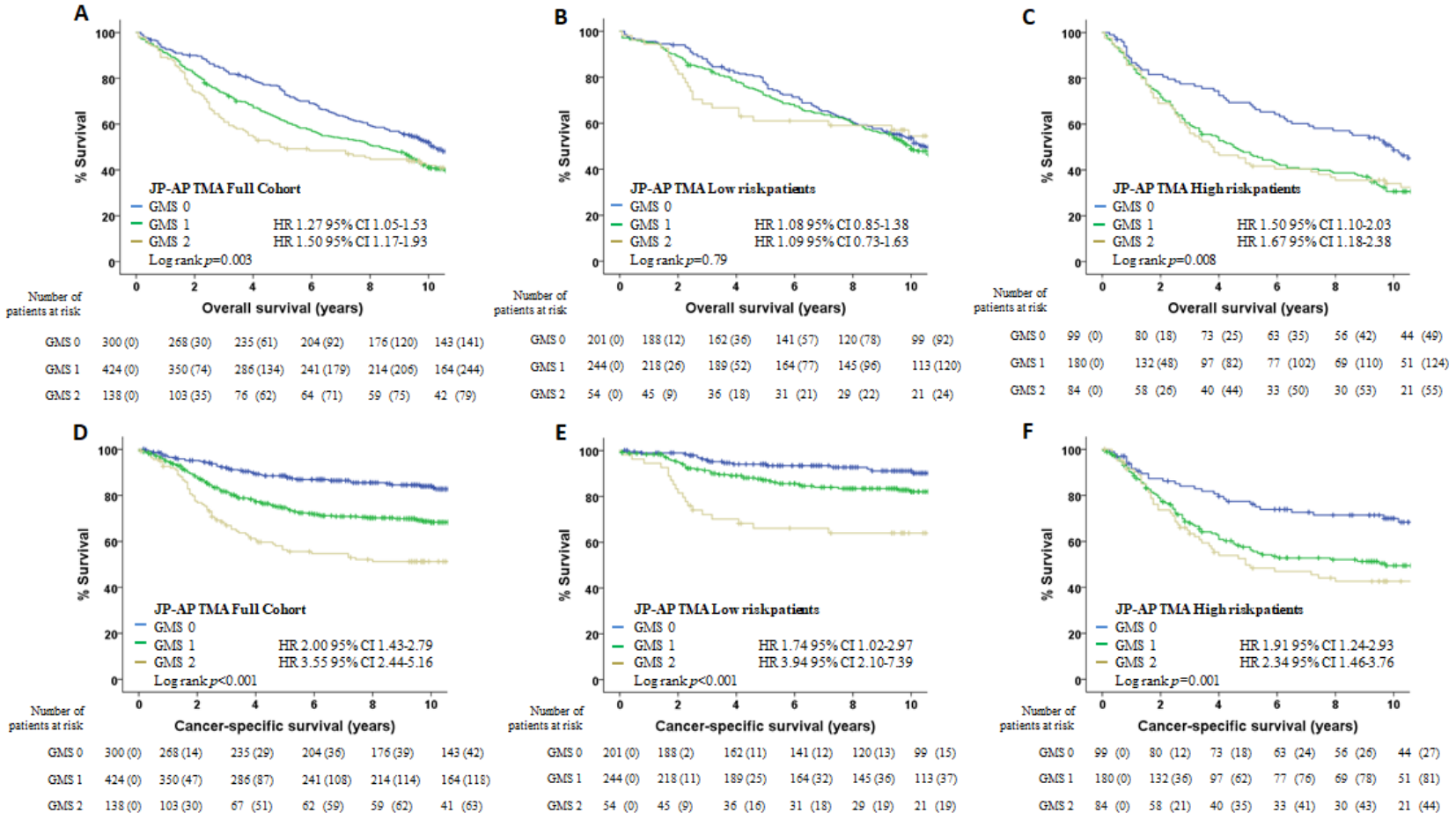


Figure 4.7. GMS and overall and cancer-specific survival according to disease risk in JP-AP TMA cohort. (A-C) GMS and OS in (A) full cohort (n=862), (B) "low-risk" CRC (n=499) and (C) "high-risk" CRC (n=363). (D-F) GMS and CSS in (D) full cohort (n=862), (E) "low-risk" CRC (n=499) and (F) "high-risk" CRC (n=363).

Overall (OS) and cancer-specific survival (CSS) data were available for the JP-AP TMA cohort and these are displayed in Tables 4.13 and 4.14 and Figure 4.7. GMS was independently significant on multivariate analysis for OS (p<0.01) and for CSS (p<0.001). On subgroup analysis for OS, the results were comparable to DFS, with GMS stratifying OS for the full cohort (GMS 0 vs GMS 2: HR 1.50 95% CI 1.17-1.93, p=0.003), high-risk disease (GMS 0 vs GMS 2: HR 1.67 95% CI 1.18-2.38, p=0.009), and colon cancer (GMS 0 vs GMS 2: HR 1.49 95% CI 1.11-2.00, p=0.02), but not low-risk disease or rectal cancer. Likewise, the subgroup analysis for CSS was similar to that for RFS, with GMS stratifying CSS for the full cohort (GMS 0 vs GMS 2: HR 3.55 95% CI 2.44-5.16, p<0.001), low-risk disease (GMS 0 vs GMS 2: HR 3.94 95% CI 2.10-7.39, p<0.001), high-risk disease (GMS 0 vs GMS 2: HR 2.34 95% CI 1.46-3.76, p=0.001), colon cancer (GMS 0 vs GMS 2: HR 3.36 95% CI 2.14-5.27, p<0.001) and rectal cancer (GMS 0 vs GMS 2: HR 4.07 95% CI 2.08-7.96, p<0.001).

The relationship between GMS and pattern of recurrence was examined (Table 4.15). GMS 1 and 2 were associated with higher risk of recurrence (GMS 0 - 15%, GMS 1 – 26%, GMS 2 – 41%, p<0.001.) Although this was predominantly due to an increase in risk of distant recurrence, patients with GMS 2 were more likely to develop local recurrence compared to GMS 0 or 1.

Table 4.15. GMS and recurrence location in the JP-AP TMA (n=833).

Recurrence location		**GMS**						
	N	**0** (n=293) *N* (%)[a]		**1** (n=424) *N* (%)		**2** (n=138) *N* (%)		*Pearson* X^2
None	630	250	(85)	304	(74)	76	(59)	**<0.001**
Local	35	8	(3)	10	(2)	17	(13)	
Distant	168	35	(12)	97	(24)	36	(28)	

[a]total percentage may not equal 100 as rounded to nearest whole number

Furthermore, associations between GMS and CD3, CD8 and composite CD3/CD8 score were assessed (Table 4.16, n=208). GMS was associated with individual T-cell densities in all locations and composite score, with highest density observed in GMS 0 and lowest density generally observed in GMS 2. Univariate survival analysis found comparable hazard ratios and confidence intervals for all immune cell markers. These were not combined in multivariate analysis as all included analysis of an inflammatory variable and would therefore be mutually exclusive (see findings for inflammatory assessment meta-analysis in chapter 1).

Table 4.16. Univariate RFS for immune cell densities per tumour location and associations between GMS and CD3, CD8 and composite CD3/CD8 score in the JP TMA (*N*=208).

Group **GMS category**		**Relapse-free Survival**				**GMS**						
	N	5-year RFS (%; SE)	Events (*N*=67)	HR (95% CI)	*P*	**0** (n=74) *N* (%)[a]		**1** (n=96) *N* (%)		**2** (n=38) *N* (%)		*Pearson* X^2
GMS				*Trend*	*<0.001*							
0	74	81 (5)	16	0.38 (0.33-0.67)	**0.001**	–	–	–	–	–	–	
1	96	72 (5)	29	0.27 (0.14-0.51)	**<0.001**	–	–	–	–	–	–	
2	38	42 (8)	22	1.0 (reference)		–	–	–	–	–	–	
CD3 Invasive margin				*Trend*	*<0.001*							
Low	117	59 (5)	49	1.0 (reference)		17	(23)	75	(78)	25	(66)	**<0.001**
High	91	84 (4)	18	0.38 (0.22-0.65)	**<0.001**	57	(77)	21	(22)	13	(34)	
CD3 Stroma				*Trend*	*<0.001*							
Low	106	57 (5)	48	1.0 (reference)		25	(34)	57	(59)	24	(63)	**<0.001**
High	102	83 (4)	19	0.33 (0.19-0.56)	**<0.001**	49	(66)	39	(41)	14	(37)	
CD3 Cancer nests				*Trend*	*<0.001*							
Low	139	60 (4)	60	1.0 (reference)		33	(45)	71	(74)	35	(92)	**<0.001**
High	69	90 (4)	7	0.18 (0.08-0.39)	**<0.001**	41	(55)	25	(26)	3	(8)	
CD8 Invasive margin				*Trend*	*0.001*							
Low	122	62 (5)	49	1.0 (reference)		28	(38)	69	(72)	25	(66)	**<0.001**
High	86	82 (4)	18	0.43 (0.25-0.74)	**0.002**	46	(62)	27	(28)	13	(34)	

CD8 Stroma				*Trend*	*0.001*							
Low	157	64 (4)	60	1.0 (reference)		45	(61)	82	(85)	30	(79)	**0.006**
High	51	88 (5)	7	0.30 (0.14-0.66)	**0.003**	29	(39)	14	(15)	8	(21)	
CD8 Cancer nests				*Trend*	*<0.001*							
Low	149	61 (4)	61	1.0 (reference)		42	(57)	72	(75)	35	(92)	**<0.001**
High	59	93 (4)	6	0.19 (0.08-0.45)	**<0.001**	32	(43)	24	(25)	3	(8)	
Composite CD3/CD8 score				*Trend*	*<0.001*							
0	72	55 (6)	33	1.0 (reference)		10	(14)	48	(50)	14	(37)	**<0.001**
1	36	55 (9)	16	0.96 (0.53-1.74)	0.88	6	(8)	20	(21)	10	(26)	
2	34	82 (7)	8	0.43 (0.20-0.93)	**0.03**	17	(23)	11	(12)	6	(16)	
3	38	79 (7)	10	0.43 (0.21-0.88)	**0.02**	23	(31)	9	(9)	6	(16)	
4	28	NA	0	NA	NA	18	(24)	8	(8)	2	(5)	

Abbreviation: RFS, Relapse-free survival; HR, hazard ratio; CI, confidence interval;

NA, not applicable (incalculable as no terminal events in this category)

[a]total percentage may not equal 100 as rounded to nearest whole number

Bold indicates $p<0.05$

The relationship between GMS and clinicopathological characteristics was examined (Table 4.11). Increasing GMS was significantly associated with younger age (p=0.04), emergency presentation (p=0.002), high-risk TNM (p<0.001), higher T- and N-stage (both p<0.001), peritoneal involvement (p<0.001) and venous invasion (p<0.001). There were no significant associations between GMS and KRAS or BRAF mutations. Neither were these mutations significant for survival in the JP-AP TMA for those with results available for analysis.

4.4.2 Implications of Results in the JP-AP TMA cohort

Since patients from the original GMS paper(Park et al., 2015) were included in this analysis this does not represent a true validation group, but an expanded analysis was able to be performed with a nearly 3-fold increase in the number of cancers. This cohort also included both colon and rectal cancers as in the original study. This expanded analysis confirmed the findings of the original study with 3 distinct bands of survival being demonstrated by GMS 0, 1 and 2 repeatedly in all forms of survival analysis.

However, the greater value of this expanded cohort was in its ability to allow subgroup analysis for greater numbers. GMS was shown to stratify DFS, RFS, OS and CSS for High-risk disease and colon cancers, and was also able to stratify RFS and CSS for low-risk disease and rectal cancers.

Furthermore, for the limited numbers where there were data available regarding K-ras and BRAF mutations, there was no evidence of any interaction with GMS.

Finally, this cohort was able to demonstrate significant trends between GMS and immunohistochemical analysis for T-cell subsets (CD3 and CD8) as well as a composite CD3/CD8 score, further emphasising the validity of KM assessment as a measurement of the immune phenotype, in agreement with the findings from the meta-analysis of immune cells in colorectal cancer (Chapter 1).

4.5 VALIDATING THE GMS IN THE TRANSSCOT COHORT

The data for the following cohort were published in the British Journal of Cancer(Alexander et al., 2020c). In order to validate the GMS in a large and independent cohort with low levels of bias given that clinicopathological data was obtained as part of a clinical trial, GMS and survival was assessed in the TransScot cohort. As has already been described in sections 2.2 and 4.1, these patients were a subgroup of those in the SCOT arm of the IDEA trial for whom pathological samples were available for analysis.

4.5.1 Results of survival analysis in the TransScot cohort

In the TransSCOT cohort, there were 2912 TNM II-III patients, all of whom received FOLFOX (*n*=846) or CAPOX (*n*=2066) adjuvant chemotherapy for at least 3 months. 383 (13%) patients were GMS 0, 1866 (64%) patients GMS 1, and 663 (23%) patients GMS 2. Median follow up was 3.0 years (range: 0.0-7.0) with 755 DFS events. Cohort characteristics shown in Table 4.17 were similar to those in the full SCOT trial and therefore representative of this population(Iveson et al., 2018).

In the full cohort, GMS significantly stratified survival with a 5-year DFS for GMS 0, 1 and 2 of 69%, 63% and 53%, respectively (GMS 0 vs GMS 2: HR 1.68 95% CI 1.28-2.20, $p<0.001$, Figure 4.8A). Patients were then assessed according to disease site. In patients with colon cancer (*n*=2402), GMS stratified survival, with 5-year DFS for GMS 0, 1 and 2 of 76%, 66% and 56%, respectively (GMS 0 vs GMS 2: HR 2.20 95% CI 1.64-2.94, $p<0.001$, Figure 4.8B). For patients with rectal cancer (*n*=510), GMS did not associate with DFS (GMS 0 vs GMS 2: HR 1.74 95% CI 0.85-3.57, $p=0.130$, Figure 4.8C). On multivariate analysis (Table 4.17), T-stage ($p<0.001$), N-stage ($p<0.001$) and GMS ($p<0.001$) independently associated with DFS. Furthermore, GMS associated with higher T-stage ($p<0.001$), higher N-stage ($p=0.002$), colonic site ($p=0.021$) and higher-risk TNM III disease ($p<0.001$).

Table 4.17. Disease-free survival in the TransSCOT cohort and associations of clinicopathological features with GMS (*N*=2912).

Clinicopathological characteristics		**Disease-free survival**				**GMS category**						
	N (%)[a]	Univariate HR (95% CI)	*P*	Multivariate HR (95% CI)	*P*	**0** (n=383) *N* (%)[a]		**1** (n=1866) *N* (%)		**2** (n=663) *N* (%)		*Pearson* X^2
Gender												
Female	1135 (39)					156	(41)	716	(38)	263	(40)	0.63
Male	1777 (61)	0.96 (0.83-1.11)	0.60	-	-	227	(59)	1150	(62)	400	(60)	
T-stage												
T1	78 (3)					19	(5)	55	(3)	4	(1)	**<0.001**
T2	250 (9)					59	(15)	160	(9)	31	(5)	
T3	1695 (58)					227	(60)	1130	(61)	338	(51)	
T4	889 (30)	1.70 (1.51-1.91)	**<0.001**	1.74 (1.53-1.98)	**<0.001**	78	(20)	521	(28)	290	(43)	
N-stage												
N0	556 (19)					79	(21)	362	(19)	115	(17)	**0.002**
N1	1663 (57)					224	(58)	1086	(58)	353	(53)	
N2	693 (24)	1.75 (1.57-1.96)	**<0.001**	1.73 (1.48-2.03)	<**0.001**	80	(21)	418	(22)	195	(29)	
Site												
Colon	2402 (82)					310	(81)	1522	(81)	570	(86)	**0.021**
Rectum	510 (18)	0.69 (0.56-0.85)	**<0.001**	-	0.12	73	(19)	344	(19)	93	(14)	
Risk Group												
T1-3/N1 (lower-risk)	1284 (55)					202	(66)	861	(57)	221	(40)	**<0.001**
T4 and/or N2 (higher-risk)	1072 (45)	2.45 (2.08-2.88)	**<0.001**	–	0.13	102	(34)	643	(43)	327	(60)	
Adjuvant therapy												
FOLFOX	846 (29)					120	(31)	526	(28)	200	(30)	0.36
CAPOX	2066 (71)	1.08 (0.92-1.27)	0.32	-	-	263	(69)	1340	(72)	463	(70)	

Treatment time												
3 months	1468 (50)					194	(51)	955	(51)	319	(48)	0.39
6 months	1444 (50)	1.01 (0.88-1.16)	0.85	-	-	180	(49)	911	(49)	344	(52)	
GMS												
0	383 (13)					**-**		**-**		**-**		**-**
1	1866 (64)					**-**		**-**		**-**		
2	663 (23)	1.48 (1.32-1.68)	**<0.001**	1.28 (1.12-1.47)	<**0.001**	**-**		**-**		**-**		

[a]percentages rounded to nearest whole number and may not total 100%

Bold indicates $p<0.05$

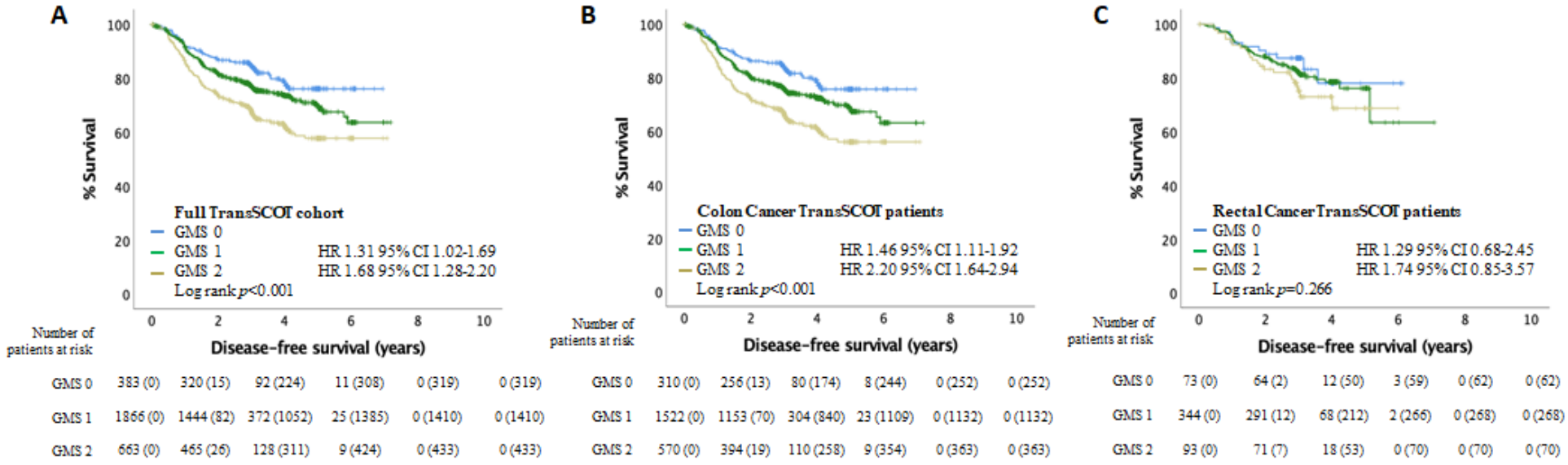


Figure 4.8. GMS and disease-free survival according to location of cancer in the TransSCOT cohort. (A) GMS and DFS in the full cohort (*n*=2912); (B) GMS in colon cancer (*n*=2402); (C) GMS in rectal cancer (*n*=510).

The utility of GMS was next assessed in lower- and higher-risk TNM III disease, as defined by the SCOT trial, TNM III patients were stratified into lower-risk (T1-3/N1) and higher-risk (T4 or N2) groups. GMS was not able to stratify DFS in the lower-risk patients as a whole, but there was still a significant survival difference between GMS 0 and 2 (GMS 0 vs GMS 2: HR 1.61 95% CI 1.01-2.57, p=0.13, Figure 4.9A). GMS was, however, able to stratify higher-risk patients (GMS 0 vs GMS 2: HR 1.86 95% CI 1.26-2.76, p=0.002, Figure 4.9B).

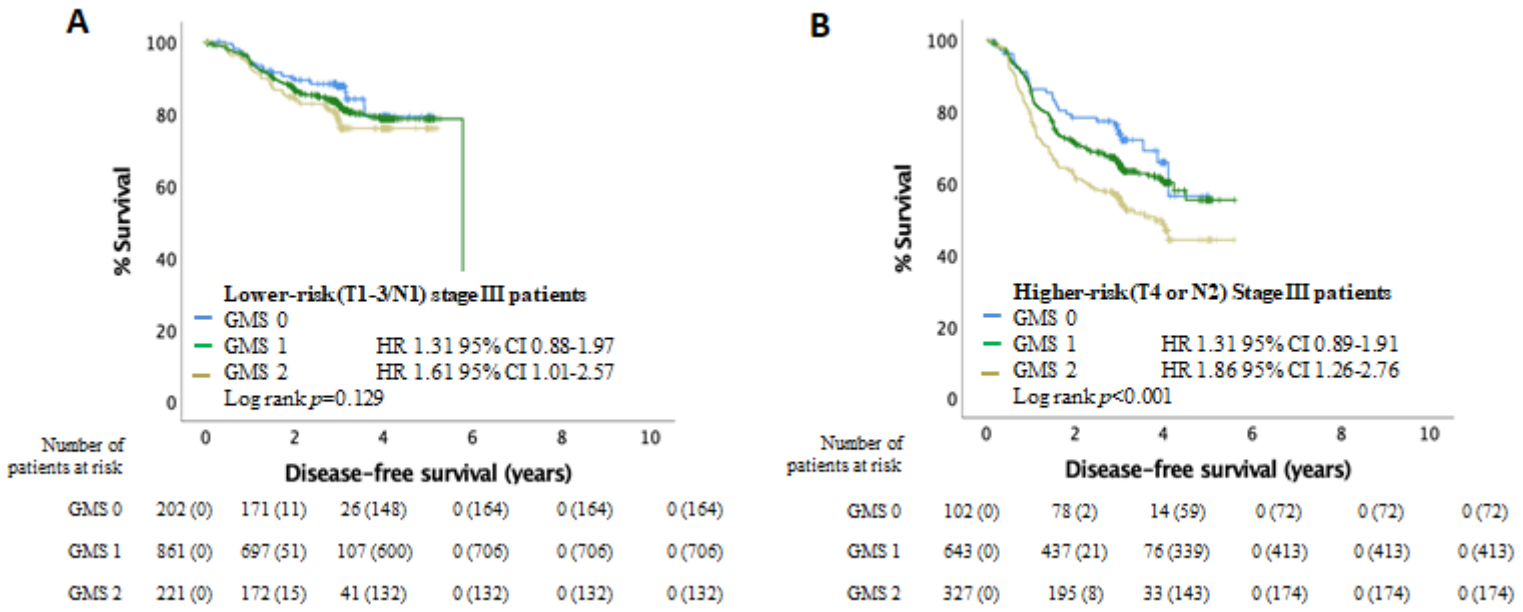


Figure 4.9. GMS and DFS in lower- and higher-risk stage III patients from the TransSCOT cohort (n=2356). (A) lower-risk stage III (n=1284); (B) higher-risk stage III (n=1072).

4.5.2 Implications of Results in the TransScot cohort

Since this data comes from a truly independent cohort from the context of a clinical trial, much of the bias that is inherent outside of a clinical trial setting is eliminated, giving an excellent opportunity to validate the GMS. In this cohort, only Disease-free Survival was available. The DFS curves for GMS in the TransScot cohort were similar to those witnessed in the combined JP-AP cohort. GMS was independently significant on multivariate analysis of the whole cohort. The significance of GMS was maintained in colon cancer, but GMS was unable to stratify DFS for rectal cancer in the TransScot cohort. The same findings were seen for DFS in the combined JP-AP cohort for rectal cancer. This cohort, made up entirely of stage III patients, was separated into a lower and higher risk group with lower-risk defined as T1-3 and N1 vs T4 or N2 disease for higher-risk. For both analyses, GMS 2 disease had a significantly worse prognosis compared with GMS 0, although the survival bands were not a clearly defined as in the full cohort analysis. When similar analysis was performed for Overall Survival in the combined JP-AP cohort, there was no significant difference in survival in the low-risk group (defined there as TNM I-II and Petersen index <2), whereas in the high-risk group (defined as TNM III or TNM II with Petersen index ≥2) GMS 0 defined a group with significantly better prognosis than GMS 1 or 2.

The validation of GMS once again highlights the significantly worse outcomes for patients with GMS 2 and emphasises the importance of following these patients up more intensively than other GMS categories, especially low-risk GMS 0.

4.6 DISCUSSION

The cohorts displayed in this chapter represent an internal validation of the results from the original study by Park et al(Park et al., 2015). GMS consistently stratified colon and colorectal cancer survival independent of other clinicopathological features. Typically, survival was separated into three distinct bands with GMS 0 having the best prognosis, GMS 2 having the worst prognosis and GMS 1 having an intermediate prognosis, depending on the cohort, survival type and clinicopathological subgroup being assessed.

The validation of the GMS in the AP-TMA cohort exposed some potential confounding factors arising from the retrospectively curated nature of this cohort's construction. In particular, the greatly increased numbers of emergency colorectal cancers identified among the colon cancer subgroup. It is likely that this feature of the dataset manifested the unexpected decline in the OS and node-positive survival curves of the GMS 1 subgroup. An alternative hypothesis was that the removal of the rectal cancers may have led to the difference in results and this was explored in the study that followed. Nevertheless, GMS was independently significance in this subgroup and GMS was stratified into three distinct survival bands.

The GRI-CRC-TMA cohort, being prospectively collated, did not suffer from the same confounders as the AP-TMA and the number of emergency presentations for colon cancer was at an expected level. The GMS in this purely colonic cohort was able to stratify survival in the same manner as the original GMS paper(Park et al., 2015). It was concluded, therefore, that the removal of the rectal cancers was not responsible for difference in GMS 1 survival in the AP-TMA analysis. The retrospective construction and higher emergency presentations was likely to be responsible for these unexpected findings.

The combined JP-AP TMA cohort did not represent a true validation cohort since it included some of the patients from the original study. However, this expanded cohort had greater

value in its ability to further examine GMS in different clinicopathological subgroups, finding that RFS and CSS were stratified by GMS for all subgroups. DFS and OS were also stratified by GMS, but only for colon cancers and high-risk groups. The further benefit of this cohort was the data available on immunohistochemistry for immune cell subtypes, which were shown to correlate well with GMS subgroups, higher in GMS 0 and lower in GMS 2. This finding of strong KM associating with immune cell subtypes was also shown by another group(Väyrynen et al., 2013). Furthermore, data was available for K-RAS and BRAF for 212 patients in this cohort, although these were neither found to be significant for survival, nor associated with GMS category. GMS stratified CSS of both low-risk and high-risk patients, in terms of stage and Petersen index, with GMS 2 highlighting a group of patients in the clinically low-risk category that may benefit from more intensive surveillance and possibly from additional adjuvant therapy. GMS 2 might therefore be considered for addition to the current list of high-risk pathological features discussed at multidisciplinary team meetings to guide ongoing management. GMS 1 in the JP-AP cohort defined a group of patients with neither strong immunity, nor high TSP who have an intermediate outcome that varies with disease stage, with better survival in low-risk disease, but worse survival in high-risk disease. Whereas, GMS 0 indicated a group of patients that had a good clinical outcome regardless of disease stage, in keeping with previous research in high immune tumours(Alexander et al., 2020b).

Patients with GMS 2 reflect a particularly poor prognostic group, with a clear reduction in not only OS, but also DFS, CSS and RFS. Previous work has proposed that such a phenotype, characterised by high stromal infiltration and weak immune response, reflects a mesenchymal subtype with poor prognosis and increased risk of recurrence(Roseweir et al., 2020).

Finally, in an independent cohort of nearly 3000 patients (TransSCOT cohort) from a clinical trial context and therefore with minimal bias, the GMS was found to be associated with DFS

in the full cohort and colon cancers, as with the combined JP-AP TMA cohort. GMS was unable to stratify survival in rectal cancers according to DFS. When TNM III disease was split into higher- and lower-risk categories, GMS 2 had significantly worse DFS than GMS 0 for both subgroups.

This study represented the largest study to date investigating a combination scoring system of peritumoural inflammation and mesenchymal phenotype. Other microenvironment scores have been proposed, such as: the Immunoscore(Pagès et al., 2018), which uses immunohistochemical staining for CD3 and CD8 and a digital pathology software platform to evaluate immune infiltrates (described more fully in section 1.5.4.6); colorectal cancer intrinsic subtypes (CRIS), which uses genetic testing of a number of genes implicated in colorectal cancer to stratify tumour behaviour/response(Isella et al., 2017); the Phenotypic Subtypes, which have already been addressed in this chapter, combining KM, TSP and Ki67 immunohistochemistry; and the image-based consensus molecular subtype, which uses artificial intelligence analysis of digital pathology slides(Sirinukunwattana et al., 2019). GMS has advantages over these scores in that it does not require the use of additional immunohistochemical staining, genetic testing or digital pathology, as it can be performed on the H&E slides that are used in routine clinical practice for TNM staging. Furthermore, in the subset of patients with both GMS and IHC available, GMS was strongly associated with CD3 and CD8. In addition, there were similar univariate RFS for all scores inflammatory scores in Table 4.16. This again supports the GMS as a clinically applicable prognostic score in patients with colorectal cancer.

Finally, GMS was unable to significantly stratify DFS or OS of patients with rectal cancer in any cohort. There were smaller numbers in this subgroup and this may be one reason for the lack of stratification. In addition, a proportion of patients may have received neoadjuvant radiotherapy, which would impact upon post-operative tumour microenvironment assessment. However, there were significant differences in survival between GMS 0 vs GMS

2 for both RFS and CSS in the JP-AP TMA cohort; this requires further study in additional patient cohorts.

In summary, all of these cohorts validate the prognostic utility of the Glasgow Microenvironment Score, particularly in the context of colon cancers. The poor outcome of GMS 2, even in the context of otherwise low-risk disease (TNM II and Petersen index<2), indicates that this subgroup should be considered an additional high-risk feature that warrants consideration for more intense follow up and possibly for adjuvant therapy. Conversely, the GMS 0 subgroup appear to have a better outcome, even in high-risk patients. GMS should be further assessed in the context of prospective randomised clinical trials.

Since the GMS had been shown to be an effective pathological scoring system in stratifying colorectal cancer survival, the next question to be posed was its utility and applicability to clinical practice. Could the GMS be useful in identifying individuals who were at greater risk of cancer relapse? Did specific GMS categories indicate potential locations of cancer recurrence? What were the effects of GMS on patients receiving specific chemotherapy regimens?

5. GMS AND CRC RECURRENCE

The clinical utility of the GMS was subsequently assessed in the GRI-CRC-TMA, for which data was available on recurrence location for many of the patients. The main question asked initially was whether GMS would be able to specify location and likelihood of recurrence for those patients in each category.

Previous studies have suggested that high stromal tumours, represented by GMS 2, have a higher rate of local recurrence(van Gestel et al., 2014, Hutchins et al., 2018). Given that prognosis is good in those with strong peritumoural inflammation, represented by GMS 0, it was hypothesised that patients in this group would have a low recurrence rate in general. GMS 1 represents a heterogenous group with neither strong peritumoural inflammation nor high TSP with an anticipated intermediate recurrence rate. Therefore, GMS may select patients who are more at risk of disease recurrence and who, as a result, may benefit from more intense postoperative surveillance.

Furthermore, since a distinction began to be seen between colon cancer and rectal cancer in terms of GMS and survival, the two broad disease sites were also assessed separately with regard to survival and recurrence. In the previously assessed JP-TMA cohort, there was evidence of GMS stratification of CSS and RFS in rectal cancer (Chapter 4.4), whilst thus far, survival according to GMS in the GRI-CRC-TMA had only been assessed in colon cancer (Chapter 4.3).

5.1 SPECIFIC METHODS

5.1.1 Patient cohort

The GRI-CRC TMA was used to explore the recurrence rates and sites for patients in each GMS category. As previously described, 1000 patients having colorectal cancers resected between January 1997 and May 2013 in Glasgow Royal Infirmary(Park et al., 2016b). The following exclusions were applied: thirty-day mortality, TNM 4 disease, and R1 resection (positive resection margins). Of the remaining 906 patients, pathology samples were available for 783 tumours. The primary endpoints were cancer-specific survival (CSS), defined as time from surgery to death from colorectal cancer and overall survival (OS), defined as time from surgery to date of death from any cause. Survival data were available until the 1st July 2020. Data on location of recurrence were collected, and for the purpose of multivariate Cox regression analysis of recurrence, the time from date of surgery to date of radiological or pathological confirmation of recurrence was used.

5.1.2 Clinicopathological characteristics

Clinical characteristics and recurrence data were recorded from patient case notes, both paper and electronic, and site of recurrence from imaging. Pathological data, including TNM stage and venous invasion (using elastic H&E-staining, for which both intra- and extramural venous invasion was considered as present) were collected from pathology reports. As previously described(Park et al., 2016b), the modified Glasgow Prognostic Score was calculated using CRP (C-reactive Protein) and Albumin levels in whole venous blood obtained within the 30 days preceding surgery. Data was available regarding which patients received adjuvant chemotherapy, but not the regimen or duration of chemotherapy. The Petersen index was used to indicate low- and high-risk TNM stage II disease(Petersen et al., 2002): tumours with venous invasion or peritoneal involvement were assigned a score of 1, whereas tumour perforation was assigned a score of 2. Any individual with TNM III disease

or TNM II with a Petersen index ≥2 was considered high-risk. The definition of emergency surgery was unplanned surgery on index hospital admission within 5 days.

5.1.3 GMS scoring

A detailed description has already been given in the Generic Methods chapter (sections 2.4.1 and 2.4.2) regarding the scoring of Klintrup-Mäkinen grade (KM) and Tumour Stroma Percentage (TSP). Scores were performed by PGA. Fifty cases were co-scored by a second investigator (HCvW) and for all scores, intra-class correlation co-efficient was >0.8.

These were combined as the GMS as previously described(Alexander et al., 2020c). In brief, strong KM and any TSP scored GMS 0; weak KM with low TSP scored GMS 1 and weak KM with high TSP scored GMS 2.

5.1.4 Statistical analysis

All data were analysed using SPSS version 27.0 (IBM SPSS). Survival analysis was performed using Kaplan-Meier curves and log-rank analysis with adjustment for T-stage, N-stage and other clinicopathological features, where appropriate. Results are presented with hazard ratios (HR) and 95% confidence intervals (CI) calculated with univariate Cox regression analysis. Multivariate survival analysis was performed using a backward conditional stepwise model. A statistical significance threshold of $p<0.1$ was used to identify variables for inclusion in the multivariate model. In-text results are given as HR, 95% CI for GMS 0 vs GMS 2, *p*-value of log-rank analysis for overall trend. Chi-squared analysis was performed to test associations between categorical variables and GMS. The study conformed to the REMARK guidelines(McShane et al., 2005) and statistical significance value was set at $p<0.05$.

5.2 RESULTS

Samples were available for 783 tumours, out of a possible 906, with TNM I-III CRC. Compared with the missing samples, those with H&E-stained slides available were more likely to have higher T-stage, more venous invasion and to be colonic rather than rectal location (Table 5.1). Clinicopathological characteristics for included patients are given in Table 5.2. Sixty-seven percent of patients were younger than 75 years at time of surgery; 55% were male; 8% presented as an emergency and 61% were node-negative. One hundred and thirty-two patients (17%) were GMS 0; 501 (64%) were GMS 1 and 149 (19%) were GMS 2. There were 477 deaths, of which 201 were related to CRC, and 221 developed recurrence. Of the recurrences, 66 patients developed local recurrence with or without systemic recurrence. An increasing GMS was associated with emergency presentation (p=0.04), higher T- and N-stage (both p<0.001) and venous invasion (p<0.001), (Table 5.2).

Table 5.1. Clinicopathological variables for patients with no H&E-stained slides (*N*=123) vs those with H&E-stained slides (*N*=783).

Clinicopathological characteristics	**No H&E available**	**H&E available**	**Chi square**
	N (%)[a]	*N* (%)[a]	*P*
Age			
≤64	46 (37)	257 (33)	0.27
65-74	41 (33)	265 (34)	
≥75	36 (29)	261 (33)	
Gender			
Female	59 (48)	354 (45)	0.57
Male	64 (52)	429 (55)	
Presentation			
Elective	113 (92)	719 (92)	0.99
Emergency	10 (8)	64 (8)	
TNM			
I	34 (28)	112 (14)	**0.006**
II (low-risk)	48 (39)	368 (47)	
III (high-risk)	41 (33)	303 (39)	
T-stage			
T1	22 (18)	43 (6)	**<0.001**
T2	17 (12)	92 (12)	
T3	65 (53)	451 (58)	
T4	19 (15)	197 (25)	
N-stage			
N0	82 (67)	480 (61)	0.47
N1	28 (23)	225 (29)	
N2	13 (11)	78 (10)	
Site			
Colon	68 (55)	554 (71)	**0.001**
Rectum	55 (45)	229 (29)	
Differentiation			
Well/mod	112 (93)	705 (91)	0.47
Poor	9 (7)	74 (9)	
Venous invasion			
Absent	71 (58)	374 (48)	**0.04**
Present	52 (42)	409 (52)	
mGPS			
0	83 (67)	500 (64)	0.45
1	23 (19)	160 (20)	
2	17 (14)	123 (16)	
GMS			
0	-	132 (17)	-
1	-	501 (64)	
2	-	150 (19)	

[a]percentages rounded to nearest whole number and may not total 100%

Table 5.2. Cancer-specific and overall survival in stage I-III colorectal cancer and associations of clinicopathological features with GMS

		Cancer-specific survival				Overall survival				GMS category						
Clinico-pathological characteristics	*N* (%)[a]	Univariate HR (95% CI)	*P*	Multivariate HR (95% CI)	*P*	Univariate HR (95% CI)	*P*	Multivariate HR (95% CI)	*P*	**0** (n=132) *N* (%)[a]		**1** (n=501) *N* (%)		**2** (n=150) *N* (%)		X^2
Age																
≤64	257 (33)									50	(38)	162	(32)	45	(30)	0.72
65-74	265 (34)									34	(26)	174	(35)	57	(38)	
≥75	261 (33)	1.39 (1.17-1.66)	**<0.001**	1.37 (1.15-1.64)	**0.001**	1.85 (1.64-2.08)	**<0.001**	1.79 (1.59-2.02)	**<0.001**	48	(36)	165	(33)	48	(32)	
Gender																
Female	354 (45)									61	(46)	231	(46)	62	(41)	0.39
Male	429 (55)	1.16 (0.88-1.54)	0.29	–	–	1.12 (0.93-1.34)	0.23	–	–	71	(54)	270	(54)	88	(59)	
Presentation																
Elective	719 (92)									125	(95)	462	(92)	132	(88)	**0.04**
Emergency	64 (8)	2.11 (1.41-3.14)	**<0.001**	–	0.10	1.46 (1.09-1.96)	**0.012**	–	0.57	7	(5)	39	(8)	18	(12)	
TNM																
I	112 (14)									41	(31)	65	(13)	6	(4)	**<0.001**
II	368 (47)									62	(47)	249	(50)	57	(38)	
III	303 (39)	2.32 (1.83-2.93)	**<0.001**[b]	–	–	1.39 (1.21-1.59)	**<0.001**[b]	–	–	29	(22)	187	(37)	87	(58)	
T-stage																
T1	43 (6)									17	(13)	25	(5)	1	(1)	**<0.001**
T2	92 (12)									33	(25)	53	(11)	6	(4)	
T3	451 (58)									68	(52)	300	(60)	83	(55)	
T4	197 (25)	1.78 (1.44-2.19)	**<0.001**	1.35 (1.08-1.70)	**0.009**	1.33 (1.17-1.50)	**<0.001**	–	0.08	14	(11)	123	(25)	60	(40)	
N-stage																
N0	480 (61)									103	(78)	314	(63)	63	(42)	**<0.001**
N1	225 (29)									25	(19)	139	(28)	61	(41)	
N2	78 (10)	1.93 (1.61-2.31)	**<0.001**	1.77 (1.47-2.14)	**<0.001**	1.29 (1.13-1.46)	**<0.001**	1.29 (1.12-1.47)	**<0.001**	4	(3)	48	(10)	26	(17)	

Site																
Colon	554 (71)									93	(71)	359	(72)	102	(68)	0.63
Rectum	229 (29)	1.08 (0.80-1.45)	0.63	–	–	0.99 (0.81-1.20)	0.90	–	–	39	(30)	142	(28)	48	(32)	
Neoadjuvant therapy																
No	725 (93)									127	(96)	464	(93)	134	(91)	0.06
Yes	54 (7)	0.99 (0.59-1.68)	0.98	–	–	0.68 (0.45-1.03)	0.07	–	0.65	5	(4)	35	(7)	14	(10)	
Differentiation																
Well/mod	705 (91)									128	(97)	442	(89)	135	(90)	0.06
Poor	74 (9)	1.10 (0.68-1.79)	0.70	–	–	1.23 (0.91-1.66)	0.18	–	–	4	(3)	55	(11)	15	(10)	
Venous invasion																
Absent	374 (48)									74	(56)	247	(49)	53	(35)	**<0.001**
Present	409 (52)	1.48 (1.12-1.97)	**0.006**	–	0.49	1.20 (1.00-1.44)	**0.047**	–	0.43	58	(44)	254	(51)	97	(65)	
mGPS																
0	500 (64)									88	(67)	323	(65)	89	(59)	0.19
1	160 (20)									22	(17)	108	(22)	30	(20)	
2	123 (16)	1.39 (1.17-1.66)	**<0.001**	1.25 (1.04-1.51)	**0.02**	1.44 (1.28-1.61)	**<0.001**	1.28 (1.13-1.44)	**<0.001**	22	(17)	70	(14)	31	(21)	
GMS																
0	132 (17)									–		–		–		–
1	501 (64)									–		–		–		
2	150 (19)	1.95 (1.54-2.46)	**<0.001**	1.50 (1.17-1.92)	**0.001**	1.41 (1.21-1.65)	**<0.001**	1.22 (1.04-1.43)	**0.016**	–		–		–		

[a]percentages rounded to nearest whole number and may not total 100% [b]not included in multivariate model as T-stage and N-stage included separately

Bold indicates $p<0.05$

Associations between GMS and CSS were assessed (Table 5.2, Figure 5.1A). GMS was able to stratify CSS in the whole cohort with 5-year CSS of 89% for GMS 0, 78% for GMS 1 and 61% for GMS 2 (GMS 0 vs GMS 2: HR 3.72 95% CI 2.22-6.24, $p<0.001$). On multivariate analysis, GMS remained independent ($p=0.001$) of age ($p=0.001$), T-stage ($p=0.009$), N-stage ($p<0.001$) and mGPS ($p=0.02$). Subgroup analysis was performed according to TNM stage and primary tumour location (Table 5.3). GMS was able to stratify survival in early TNM I-II disease with 5-year CSS for GMS 0, 1 and 2 of 89%, 87% and 75%, respectively (GMS 0 vs GMS 2: HR 2.89 95% CI 1.42-5.85, $p=0.003$, Figure 5.1B); and TNM III disease with 5-year CSS for GMS 0, 1 and 2 of 90%, 63% and 50%, respectively (GMS 0 vs GMS 2: HR 3.36 95% CI 1.42-7.91, $p=0.006$, Figure 5.1C). In addition, GMS was able to stratify CSS regardless of the use of adjuvant chemotherapy (No adjuvant therapy: GMS 0 vs GMS 2: HR 3.33 95% CI 1.91-5.82, $p<0.001$; Adjuvant therapy: GMS 0 vs GMS 2: HR 11.54 95% CI 1.54-86.27, $p=0.02$) or the site of primary tumour (Colon cancer: GMS 0 vs GMS 2: HR 3.54 95% CI 1.92-6.51, $p<0.001$; Rectal cancer: GMS 0 vs GMS 2: HR 4.17 95% CI 1.56-11.13, $p=0.004$).

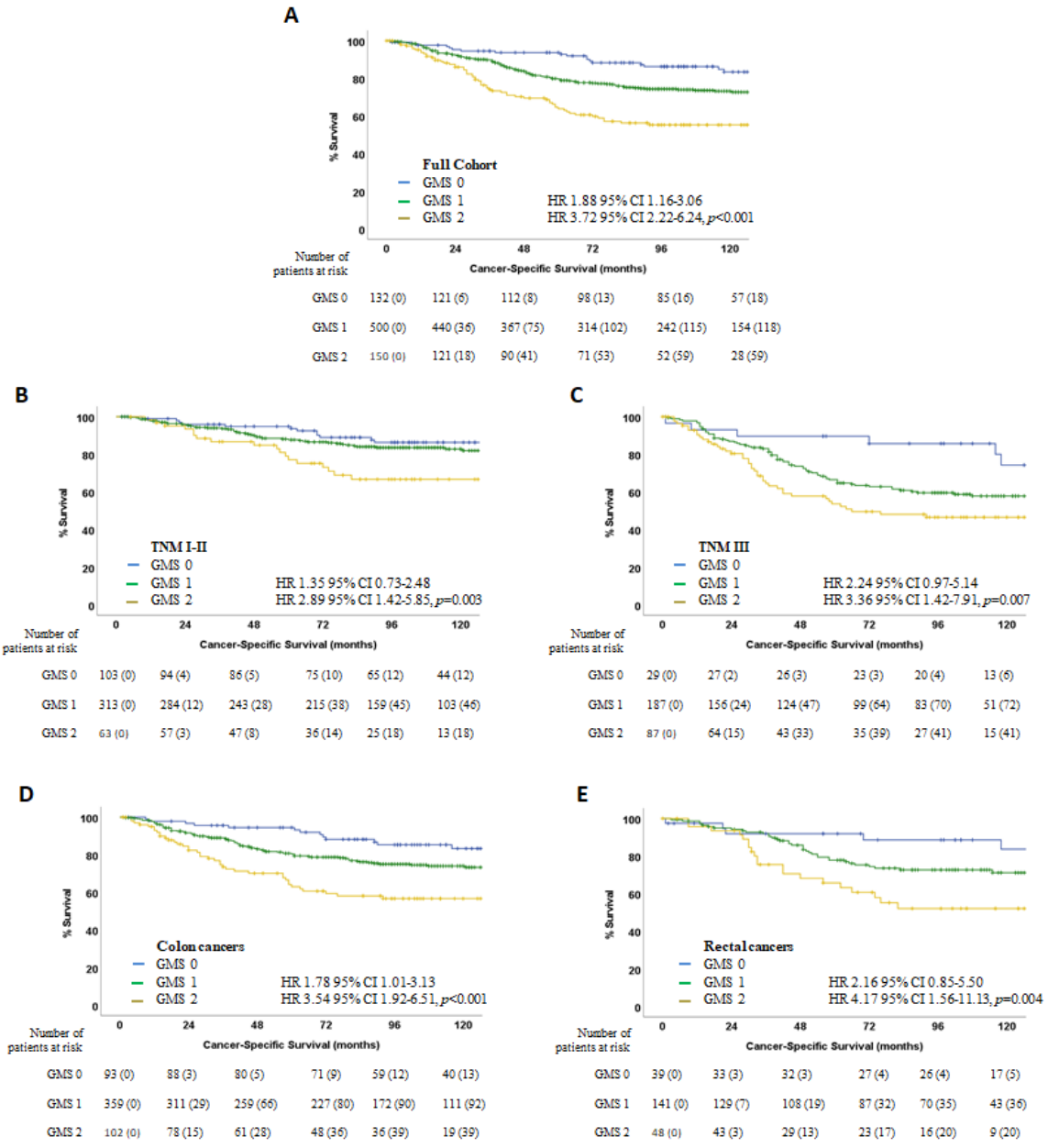


Figure 5.1. CSS according GMS in: (A) Full cohort (N=782); (B) TNM I-II (N=479); (C) TNM III (n=303); (D) Colon cancers (n=554); (E) Rectal cancers (n=228)

Table 5.3. Univariate survival analysis (CSS and OS) for GMS according to TNM, adjuvant chemotherapy and location of primary cancer

Group **GMS category**		**Cancer-specific Survival**				**Overall Survival**			
	N	5-year CSS (%; SE)	Events (*N*=201)	HR (95% CI)	*P*	5-year OS (%; SE)	Events (*N*=477)	HR (95% CI)	*P*
Full cohort				*Trend*	*<0.001*			*Trend*	*<0.001*
0	132	89 (3)	19	1.0 (reference)		75 (4)	67	1.0 (reference)	
1	501	78 (2)	122	1.88 (1.16-3.06)	**0.01**	63 (2)	310	1.40 (1.08-1.82)	**0.01**
2	149	61 (4)	60	3.72 (2.22-6.24)	**<0.001**	48 (4)	100	1.97 (1.44-2.69)	**<0.001**
TNM I-II				*Trend*	*0.003*			*Trend*	*0.09*
0	103	89 (3)	13	1.0 (reference)		74 (4)	51	1.0 (reference)	
1	314	87 (2)	50	1.35 (0.73-2.48)	0.34	69 (3)	183	1.26 (0.92-1.72)	0.15
2	63	75 (6)	19	2.89 (1.42-5.85)	**0.003**	58 (6)	38	1.59 (1.04-2.42)	**0.03**
TNM III				*Trend*	*0.007*			*Trend*	*0.006*
0	29	90 (6)	6	1.0 (reference)		79 (8)	16	1.0 (reference)	
1	187	63 (4)	72	2.24 (0.97-5.14)	0.06	54 (4)	127	1.67 (0.99-2.81)	0.05
2	86	50 (6)	41	3.36 (1.42-7.91)	**0.006**	41 (5)	62	2.32 (1.33-4.03)	**0.003**
No adjuvant chemo				*Trend*	*<0.001*			*Trend*	*0.001*
0	111	87 (3)	18	1.0 (reference)		70 (4)	63	1.0 (reference)	
1	375	78 (2)	88	1.59 (0.96-2.65)	0.07	61 (3)	252	1.33 (1.01-1.75)	**0.046**
2	99	63 (5)	40	3.33 (1.91-5.82)	**<0.001**	47 (5)	72	1.88 (1.34-2.64)	**<0.001**
Adjuvant chemo				*Trend*	*0.007*			*Trend*	*0.002*
0	21	100 (0)	1	1.0 (reference)		100 (0)	4	1.0 (reference)	
1	126	76 (4)	34	6.82 (0.93-49.86)	0.06	71 (4)	58	3.33 (1.21-9.20)	**0.02**
2	49	58 (7)	19	11.54 (1.54-86.27)	**0.02**	51 (7)	27	5.30 (1.84-15.26)	**0.002**
Colon cancer				*Trend*	*<0.001*			*Trend*	*0.002*
0	93	90 (3)	14	1.0 (reference)		77 (4)	47	1.0 (reference)	
1	359	79 (2)	85	1.78 (1.01-3.13)	**0.046**	64 (3)	225	1.40 (1.03-1.92)	**0.04**
2	102	61 (5)	40	3.54 (1.92-6.51)	**<0.001**	47 (5)	68	1.95 (1.34-2.83)	**<0.001**

Rectal Cancer				*Trend*	*0.004*			*Trend*	*0.04*
0	39	89 (5)	5	1.0 (reference)		69 (7)	20	1.0 (reference)	
1	142	75 (4)	37	2.16 (0.85-5.50)	0.11	63 (4)	85	1.38 (0.84-2.24)	0.20
2	47	61 (8)	20	4.17 (1.56-11.13)	**0.004**	51 (7)	32	2.02 (1.15-3.54)	**0.015**

Next, associations between GMS and OS were assessed (Table 5.2). GMS was able to stratify OS in the whole cohort with 5-year OS of 75% for GMS 0, 63% for GMS 1 and 48% for GMS 2 (GMS 0 vs GMS 2: HR 1.97 95% CI 1.44-2.69, $p<0.001$). On multivariate analysis, GMS was independent ($p=0.012$) of age ($p<0.001$), N-stage ($p<0.001$) and mGPS ($p<0.001$). Subgroup analysis was performed according to TNM stage and primary tumour location (Table 5.3). GMS was able to stratify survival in TNM I-II disease with 5-year OS for GMS 0, 1 and 2 of 74%, 69% and 58%, respectively (GMS 0 vs GMS 2: HR 1.59 95% CI 1.04-2.42, $p=0.03$); and TNM III disease with 5-year OS for GMS 0, 1 and 2 of 79%, 54% and 41%, respectively (GMS 0 vs GMS 2: HR 2.32 95% CI 1.33-4.03, $p=0.003$). In addition, GMS was able to stratify OS regardless of the use of adjuvant chemotherapy (No adjuvant therapy: GMS 0 vs GMS 2: HR 1.88 95% CI 1.34-2.64, $p<0.001$; Adjuvant therapy: GMS 0 vs GMS 2: HR 5.30 95% CI 1.84-15.26, $p=0.002$) or the site of primary tumour (Colon cancer: GMS 0 vs GMS 2: HR 1.95 95% CI 1.34-2.83, $p<0.001$; Rectal cancer: GMS 0 vs GMS 2: HR 2.02 95% CI 1.15-3.54, $p=0.015$).

The relationship between pattern of recurrence and GMS was subsequently examined (Table 5.4). Overall, the recurrence rate for GMS 0 was 15% during the course of follow up, compared with 27% in GMS 1 and 38% in GMS 2. The rates of local recurrence +- systemic recurrence for GMS 0, 1 and 2 were 5%, 9% and 13%, respectively ($p=0.02$). Similarly, the rates for distant recurrence only were 11%, 22% and 31%, respectively, for GMS 0, 1 and 2 ($p<0.001$). In terms of specific recurrence location, GMS 0 had the highest recurrence-free rate of 85%, vs 73% for GMS 1 and 62% for GMS 2. The numbers were small for most individual locations, but the pattern was similar for liver, lung and widespread recurrences with highest rates in GMS 2 and lowest in GMS 0.

Table 5.4. GMS and recurrence location stratified by site of primary (n=737)

Group		**GMS**						
Colon + Rectal cancer	*N*	**0** (n=125) *N* (%)[a]		**1** (n=474) *N* (%)		**2** (n=138) *N* (%)		X^2
Recurrence								
None	540	106	(85)	348	(73)	86	(62)	
Local +- systemic	66	6	(5)	42	(9)	18	(13)	**0.02**
Distant	160	14	(11)	103	(22)	43	(31)	**<0.001**
Recurrence location								
None	540	106	(85)	348	(73)	86	(62)	-[b]
Local only	35	5	(4)	22	(5)	8	(6)	
Nodal	3	1	(1)	2	(1)	0	(0)	
Liver	57	3	(2)	39	(8)	15	(11)	
Lung	22	3	(2)	12	(3)	7	(5)	
Brain	6	1	(1)	5	(1)	0	(0)	
Widespread	74	6	(5)	46	(10)	22	(16)	
Colon cancer only (n=522)	*N*	**0** (n=89) *N* (%)[a]		**1** (n=338) *N* (%)		**2** (n=95) *N* (%)		
Recurrence								
None	384	73	(86)	251	(82)	60	(72)	
Local +- systemic	47	4	(5)	31	(9)	12	(13)	0.06
Distant	109	13	(15)	68	(20)	28	(30)	**0.01**
Recurrence location								
None	384	73	(86)	251	(82)	60	(72)	-[b]
Local only	29	3	(3)	18	(5)	7	(7)	
Nodal	3	1	(1)	2	(1)	0	(0)	
Liver	35	3	(3)	21	(6)	11	(12)	
Lung	12	2	(2)	7	(2)	3	(3)	
Brain	4	1	(1)	3	(1)	0	(0)	
Widespread	56	6	(7)	36	(11)	15	(15)	
Rectal cancer only (n=215)	*N*	**0** (n=36) *N* (%)[a]		**1** (n=136) *N* (%)		**2** (n=43) *N* (%)		
Recurrence								
None	156	33	(92)	97	(71)	26	(61)	
Local +- systemic	19	2	(6)	11	(8)	6	(14)	0.17
Distant	51	1	(3)	35	(26)	15	(36)	**<0.001**
Recurrence location								
None	156	33	(92)	97	(71)	26	(61)	-[b]
Local only	7	2	(6)	4	(3)	1	(2)	
Nodal	0	-	-	-	-	-	-	
Liver	22	0	(0)	18	(13)	4	(9)	
Lung	10	1	(3)	5	(4)	4	(9)	
Brain	2	0	-	2	(2)	0	-	
Widespread	18	0	(0)	10	(7)	8	(19)	

[a]total percentage may not equal 100 as rounded to nearest whole number
[b]no statistical analysis as cells with n<6

Multivariate analysis was subsequently performed according to location of recurrence (Table 5.5). On univariate analysis, GMS was significant for local recurrence with or without systemic recurrence (p=0.003, Figure 5.2A), although this was not independent of T-stage (p=0.001) or N-stage (p=0.002) on multivariate analysis. GMS was significant on multivariate analysis for recurrence at any location (HR 1.38, 1.08-1.78, p=0.01, Figure 5.2D), independent of T-stage (p=0.02), N-stage (p<0.001), emergency presentation (p=0.15), venous invasion (p=0.07) and systemic inflammation (mGPS), (p=0.01).

Table 5.5. Local and systemic recurrence in stage I-III colorectal cancer and associations of clinicopathological features and GMS

Clinicopathological characteristics	**Recurrence at any location**				**Local recurrence (with or without systemic recurrence)**			
	Univariate HR (95% CI)	*P*	Multivariate HR (95% CI)	*P*	Univariate HR (95% CI)	*P*	Multivariate HR (95% CI)	*P*
Age								
≤64								
65-74								
≥75	1.14 (0.96-1.36)	0.13	–	–	1.25 (0.93-1.70)	0.15	–	–
Gender								
Female								
Male	1.24 (0.93-1.65)	0.14	–	–	1.03 (0.64-1.68)	0.89	–	–
Presentation								
Elective								
Emergency	2.01 (1.32-3.06)	**0.001**	–	0.15	2.27 (1.12-4.59)	**0.02**	–	0.46
TNM								
I								
II (low-risk)								
III (high-risk)	2.04 (1.62-2.56)	**<0.001**[b]	–	–	2.09 (1.41-3.11)	**<0.001**[b]	–	–
T-stage								
T1								
T2								
T3								
T4	1.75 (1.42-2.16)	**<0.001**	1.32 (1.05-1.66)	**0.02**	2.45 (1.66-3.62)	**<0.001**	2.01 (1.33-3.03)	**<0.001**
N-stage								
N0								
N1								
N2	1.83 (1.52-2.20)	**<0.001**	1.59 (1.31-1.94)	<**0.001**	1.89 (1.38-2.59)	**<0.001**	1.67 (1.20-2.32)	**0.002**

Site								
Colon								
Rectum	1.06 (0.78-1.44)	0.71	–	–	1.00 (0.59-1.71)	0.99		
Differentiation								
Well/mod								
Poor	1.19 (0.75-1.89)	0.43	–	–	1.43 (0.69-3.01)	0.34	–	–
Venous invasion								
Absent								
Present	1.72 (1.29-2.31)	**<0.001**	–	0.07	1.22 (0.75-1.98)	0.42	–	–
mGPS								
0								
1								
2	1.31 (1.09-1.56)	**0.003**	1.28 (1.06-1.55)	**0.01**	1.50 (1.11-2.02)	**0.01**	–	0.06
GMS								
0								
1								
2	1.76 (1.38-2.23)	**<0.001**	1.38 (1.08-1.78)	**0.01**	1.89 (1.25-2.85)	**0.003**	–	0.15

[a]percentages rounded to nearest whole number and may not total 100%

[b]not included in multivariate model as T-stage and N-stage included separately

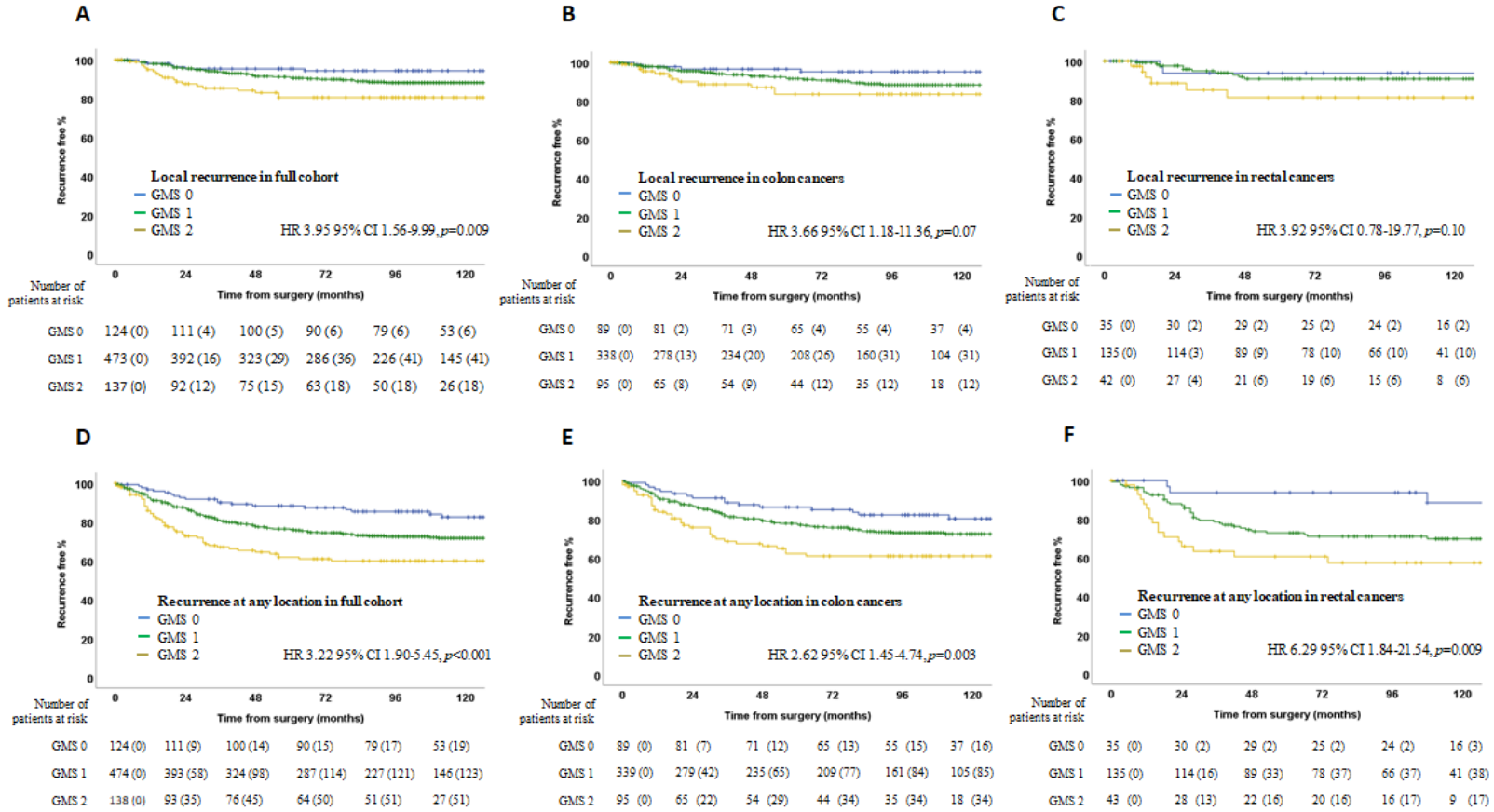


Figure 5.2. Local recurrence (+- systemic involvement), (A-C), and recurrence at any location, (D-F), stratified by GMS. (A) local recurrence in full cohort (n=783); (B) local recurrence in colon cancers (n=554); (C) local recurrence in rectal cancers (n=229); (D) recurrence at any location in full cohort; (E) recurrence at any location in colon cancers (n=554); (F) recurrence at any location in rectal cancers (n=229)

The relationship between pattern of recurrence and GMS was subsequently examined in colon and rectal cancers separately (Table 5.4, Figure 5.2). In colon cancers, the recurrence rate for GMS 0 was 20% during the course of follow up, compared with 28% in GMS 1 and 38% in GMS 2. The rates of local recurrence +- systemic recurrence for GMS 0, 1 and 2 were 5%, 9% and 13%, respectively (p=0.06, Table 5.6). Similarly, the rates for distant recurrence only were 14%, 19% and 27%, respectively, for GMS 0, 1 and 2 (p=0.04). In terms of specific recurrence location, GMS 0 had the highest recurrence-free rate of 86%, vs 82% for GMS 1 and 72% for GMS 2. The numbers were small for most individual locations, but the pattern was similar for liver, lung and widespread recurrences with highest rates in GMS 2 and lowest in GMS 0. On univariate analysis, GMS was significant for local recurrence with or without systemic recurrence (p=0.02, Figure 5.2B, Table 5.6), although this was not independent of T-stage (p=0.001) or N-stage (p=0.004) on multivariate analysis. Similarly, GMS was significant on univariate analysis for recurrence at any location (p=0.02, Figure 5.2D), although this was not independent of T-stage (p<0.001), N-stage (p<0.001) or mGPS (p=0.02).

Table 5.6. Local and systemic recurrence in stage I-III colon cancers and associations of clinicopathological features and GMS (*N*=554).

Clinicopathological characteristics	**Recurrence at any location**				**Local recurrence (with or without systemic recurrence)**			
	Univariate HR (95% CI)	*P*	Multivariate HR (95% CI)	*P*	Univariate HR (95% CI)	*P*	Multivariate HR (95% CI)	*P*
Age								
≤64								
65-74								
≥75	1.19 (0.96-1.46)	0.11	–	–	1.24 (0.87-1.78)	0.24	–	–
Gender								
Female								
Male	0.96 (0.69-1.35)	0.83	–	–	0.75 (0.42-1.33)	0.33	–	–
Presentation								
Elective								
Emergency	2.00 (1.28-3.13)	**0.002**	–	0.25	1.80 (0.81-4.02)	0.15	–	–
TNM								
I								
II (low-risk)								
III (high-risk)	2.06 (1.56-2.73)	**<0.001**[b]	–	–	2.44 (1.49-3.98)	**<0.001**[b]	–	–
T-stage								
T1								
T2								
T3								
T4	1.97 (1.52-2.57)	**<0.001**	1.65 (1.25-2.17)	<**0.001**	2.67 (1.65-4.32)	**<0.001**	2.01 (1.33-3.03)	**<0.001**
N-stage								
N0								
N1								
N2	1.78 (1.43-2.22)	**<0.001**	1.60 (1.27-2.01)	<**0.001**	2.12 (1.47-3.06)	**<0.001**	1.67 (1.20-2.32)	**0.004**

Differentiation								
Well/mod								
Poor	1.30 (0.78-2.15)	0.32	–	–	1.61 (0.72-3.60)	0.25	–	–
Venous invasion								
Absent								
Present	1.77 (1.25-2.50)	**0.001**	–	0.16	1.35 (0.76-2.41)	0.31	–	–
mGPS								
0								
1								
2	1.40 (1.13-1.72)	**0.002**	1.30 (1.05-1.62)	**0.02**	1.40 (0.98-2.00)	0.06	–	0.23
GMS								
0								
1								
2	1.63 (1.23-2.17)	**<0.001**	–	0.12	1.81 (1.11-2.94)	**0.02**	–	0.35

[a]percentages rounded to nearest whole number and may not total 100%

[b]not included in multivariate model as T-stage and N-stage included separately

Bold indicates $p<0.05$

In rectal cancers, the recurrence rate for GMS 0 was 8% during the course of follow up, compared with 29% in GMS 1 and 39% in GMS 2. The rates of local recurrence +- systemic recurrence for GMS 0, 1 and 2 were 6%, 8% and 14%, respectively (p=0.17). Similarly, the rates for distant recurrence only were 3%, 26% and 36%, respectively, for GMS 0, 1 and 2 (p<0.001). In terms of specific recurrence location, GMS 0 had the highest recurrence-free rate of 91%, vs 71% for GMS 1 and 61% for GMS 2. The numbers were small for most individual locations, but the pattern was similar for liver, lung and widespread recurrences with higher rates in GMS 2 and the lowest in GMS 0. On univariate analysis, GMS did not reach significance for local recurrence with or without systemic recurrence (p=0.06, Figure 5.2C, Table 5.7). Emergency presentation (p=0.002), T-stage (p=0.04) and mGPS (p=0.04) were significant for local recurrence in rectal cancers. There were not sufficient event numbers in this category to support multivariate analysis. GMS was significant on multivariate analysis for recurrence at any location (HR 1.99, 1.26-3.16, p=0.003, Figure 5.2F), independent of gender (p=0.006) and N-stage (p<0.001).

Table 5.7. Local and systemic recurrence in stage I-III rectal cancers and associations of clinicopathological features and GMS (*N*=229)

Clinicopathological characteristics	**Recurrence at any location**				**Local recurrence (with or without systemic recurrence)**			
	Univariate HR (95% CI)	*P*	Multivariate HR (95% CI)	*P*	Univariate HR (95% CI)	*P*	Multivariate[b] HR (95% CI)	*P*
Age								
≤64								
65-74								
≥75	1.07 (0.77-1.50)	0.68	–	–	1.27 (0.71-2.28)	0.43	–	–
Gender								
Female								
Male	2.57 (1.39-4.77)	**0.003**	2.40 (1.29-4.47)	**0.006**	2.84 (0.93-8.62)	0.07	–	–
Presentation								
Elective								
Emergency	2.85 (0.70-11.7)	0.15	–	–	10.5 (2.40-46.1)	**0.002**	–	–
TNM								
I								
II (low-risk)								
III (high-risk)	1.98 (1.33-2.94)	**<0.001**[a]	–	–	1.56 (0.81-3.02)	0.18	–	–
T-stage								
T1								
T2								
T3								
T4	1.45 (0.99-2.12)	0.05	–	0.48	2.20 (1.05-4.58)	**0.04**	–	–
N-stage								
N0								
N1								
N2	1.93 (1.37-2.70)	**<0.001**	1.81 (1.28-2.55)	<**0.001**	1.36 (0.73-2.54)	0.34	–	–

Differentiation								
Well/mod								
Poor	0.91 (0.29-2.92)	0.88	–	–	1.01 (0.13-7.56)	0.99	–	–
Venous invasion								
Absent								
Present	1.56 (0.91-2.68)	0.11	–	–	0.89 (0.36-2.21)	0.81	–	–
mGPS								
0								
1								
2	1.12 (0.76-1.64)	0.58	–	–	1.82 (1.04-3.19)	**0.04**	–	–
GMS								
0								
1								
2	2.09 (1.35-3.23)	**<0.001**	1.99 (1.26-3.16)	**0.003**	2.12 (0.97-4.64)	0.06	–	–

[a]not included in multivariate model as T-stage and N-stage included separately [b]multivariate analysis not supported for local recurrence as there were only 19 events

Bold indicates $p<0.05$

5.3 DISCUSSION

In this large, single-centre study, the GMS was observed to be an independent prognostic marker for TNM I-III CRC. Although not associated with specific site, an increasing GMS was associated with increased risk of recurrence overall.

Subgroup analysis of colon and rectal cancers individually revealed that recurrence at any location was predicted by GMS. This was independently significant in rectal cancers, although in colon cancers, was not independent of T-stage, N-stage and mGPS. The numbers of local recurrences were smaller, particularly in the rectal cancer subgroup and GMS did not reach univariate significance in this subgroup. In the colon cancer subgroup GMS was significant on univariate analysis but was not independent of T-Stage and N-stage, as shown in the full cohort.

GMS 0, characterised by higher peritumoural inflammatory response has been established as a prognostic marker conferring a survival benefit(Alexander et al., 2020b). The same effect was observed in the current study with the lowest recurrence rate in this group. It must be noted, however that the recurrence rate is not zero and whilst higher peritumoural inflammatory response is considered protective, there are clearly other factors at play in this group. Of note, the type of immune cells is not accounted for by this specific scoring system. Others have shown that polarisation of macrophages to M2 macrophages may be a poor prognostic sign(Väyrynen et al., 2021). Furthermore, the individuals that developed recurrence in spite of the beneficial phenotype of strong KM may have had a more aggressive tumour biology. These, therefore, represent areas requiring further investigation and the combination of genetic profile and GMS is one of the planned future directions of study.

In the present study, patients with GMS 2 had the highest rates both of local and distant disease recurrence. Previous work suggests that this pathological phenotype, characterised by high TSP and accompanied by a poor immune response, denotes a mesenchymal subtype

with poor prognosis and higher recurrence risk(Alexander et al., 2020c, van Gestel et al., 2014, Hutchins et al., 2018, Roseweir et al., 2020, Hansen et al., 2018). There are several confounding factors in this group with associations demonstrated between higher GMS and higher T-stage, N-stage and venous invasion, a finding also demonstrated in other studies(Hansen et al., 2018). Specifically, GMS was not independent of T-stage or N-stage, although GMS was an independent prognostic marker when comparing overall risk of recurrence at any location. This may be partly due to the lower numbers of local recurrences. Kaplan-Meier curves for both CSS and OS display an early and sustained fall in survival in the GMS 2 group.

Given the high-risk nature of the GMS 2 phenotype, these tumours may warrant more aggressive follow up with an enhanced surveillance programme, in order to detect recurrent disease at an earlier stage.

GMS has been observed to associate with both local and systemic CRC recurrence. GMS was an independent prognostic indicator for disease recurrence at any location. The numbers for local disease recurrence were low, however, and GMS was not found to be independent of T-stage and N-stage as a predictor of local disease recurrence. Nevertheless, given that GMS is a marker for recurrent colorectal cancer, patients with higher GMS tumours may benefit from enhanced postoperative surveillance to aid the earlier detection of recurrent disease.

6. GMS AND RESPONSE TO CHEMOTHERAPY

The clinical utility of the GMS was subsequently assessed in the relation to GMS and response to chemotherapy. Two cohorts were used to assess this: firstly, the GRI-CRC-TMA, for which data was available on whether any adjuvant chemotherapy was given (but not type of chemotherapy) in the group of patients that would be considered higher risk for recurrence, according to the Petersen index; secondly, the TransScot cohort was used to assess response of different GMS cohorts to type and duration of two different chemotherapeutic regimens (FOLFOX and CAPOX). The TransScot data for this chapter was published previously in the British Journal of Cancer(Alexander et al., 2020c).

Recently, a modified version of the GMS in colorectal cancer biopsy specimens (using CD3 as a marker of the immune phenotype, since it is not possible to assess KM in a biopsy specimen as the invasive margin will not be visualised) has been shown to accurately reflect that of the full resected specimen, indicating that it may be useful in dictating choice of neoadjuvant therapy(Park et al., 2020).

There are data that suggests high stromal tumours, represented by GMS 2, have a higher rate of local recurrence(van Gestel et al., 2014, Hutchins et al., 2018). This was also demonstrated in the previous chapter. It was hypothesised that GMS 2 tumours might have an inferior response to standard chemotherapy. Furthermore, since one of the main mechanisms that chemotherapy employs is the destruction of more rapidly replicating cells (i.e. tumour tissue) than healthy tissue, thereby also potentially stimulating a greater inflammatory reaction to the exposed antigens on the destroyed tissue, it was hypothesised that GMS 0 tumours might respond best to chemotherapy in general.

The GMS could provide a platform on which to develop personalized treatment approaches for CRC, which is also important for adjuvant chemotherapy, where biomarkers are lacking. For example, the SCOT trial recently demonstrated patients receiving CAPOX (capecitabine and oxaliplatin) have similar survival with 3- versus 6-months duration, whereas patients

receiving FOLFOX (bolus and infused fluorouracil with oxaliplatin) may benefit from 6-months duration(Iveson et al., 2018, Souglakos et al., 2019). Therefore, it is important to identify patients who may benefit from a longer and more intensive chemotherapy regimen. The utility of a histopathology-based classification of the Consensus Molecular Subtypes called Phenotypic Subtypes, incorporating KM grade, TSP and the proliferation marker Ki67 was recently investigated by Roseweir et al.(Roseweir et al., 2020). This stratified chemotherapy response in a cohort of 1343 patients from the adjuvant chemotherapy SCOT trial (TransSCOT), with the predictive power of this subtyping predominantly related to assessment of KM grade and TSP. Therefore, it was deemed more appropriate and pragmatic in the current study to use GMS to assess the expanded cohort in preference to Phenotypic Subtypes, since the GMS can be performed on H&E slides that are routinely used in histopathological staining without the need for immunohistochemistry.

Therefore, the primary aim of this chapter was to assess associations of GMS with adjuvant chemotherapy, type and duration in the GRI-CRC-TMA and TransSCOT cohorts.

6.1 SPECIFIC METHODS

6.1.1 Patient cohorts

The GRI-CRC TMA was used to explore the recurrence rates and sites for patients in each GMS category. As previously described, this cohort comprised 1000 patients undergoing colorectal cancer resection between January 1997 and May 2013 in Glasgow Royal Infirmary(Park et al., 2016b). The following exclusions were applied: thirty-day mortality, TNM IV disease, and R1 resection (positive resection margins). Of the remaining 906 patients, pathology samples were available for 783 tumours. The primary endpoints were cancer-specific survival (CSS), defined as time from surgery to death from colorectal cancer and overall survival (OS), defined as time from surgery to date of death from any cause. Survival data were available until the 1st July 2020.

The TransScot cohort comprised 2912 patients with available tissue from the SCOT adjuvant chemotherapy trial (ISRCTN no. 59757862) who had undergone potentially curative resection for high-risk TNM II or TNM III CRC from 2008-2013 within the UK. All patients were followed up for at least 3 years. The primary endpoint was disease-free survival (DFS; measured from date of surgery/randomization to date of recurrence or all-cause mortality) for this cohort. DFS was the only form of survival data available for study. Survival data was complete until the end of the study period for the TransSCOT cohort. Those who died within 30 days of surgery were excluded.

6.1.2 Clinicopathological characteristics

Clinical characteristics and recurrence data were recorded from patient case notes, both paper and electronic, and site of recurrence from imaging. Pathological data, including TNM stage and venous invasion (using elastic H&E-staining, for which both intra- and extramural venous invasion was considered as present) were collected from pathology reports. As previously described(Park et al., 2016b), the modified Glasgow Prognostic Score was calculated using CRP (C-reactive Protein) and Albumin levels in whole venous blood obtained within the 30 days preceding surgery. Data was available regarding which patients received adjuvant chemotherapy, but not the regimen or duration of chemotherapy. The Petersen index was used to indicate low- and high-risk TNM stage II disease(Petersen et al., 2002): tumours with venous invasion or peritoneal involvement were assigned a score of 1, whereas tumour perforation was assigned a score of 2. Any individual with TNM III disease or TNM II with a Petersen index ≥2 was considered high-risk. The definition of emergency surgery was unplanned surgery on index hospital admission within 5 days.

The clinicopathological characteristics for the TransScot cohort have been previously described(Iveson et al., 2018). Briefly, the cohort comprised of patients with stage III and high-risk stage II (one or more of T4 disease, tumour obstruction with or without perforation of the primary tumour preoperatively, fewer than ten lymph nodes

harvested, poorly differentiated histology, perineural invasion, or extramural venous or lymphatic invasion), treated with FOLFOX or CAPOX adjuvant chemotherapy randomized to 3- or 6-months' duration. Tumours were staged using 7th edition of TNM. Date and site of recurrence and cause of death were crosschecked using electronic case records for both cohorts.

6.1.3 GMS scoring

A detailed description has already been given in the Generic Methods chapter (sections 2.4.1 and 2.4.2) regarding the scoring of Klintrup-Mäkinen grade (KM) and Tumour Stroma Percentage (TSP). Scores for GRI-CRC-TMA were performed by PGA. Fifty cases were co-scored by a second investigator (HCvW) and for all scores, intra-class correlation co-efficient was >0.8. In the TransSCOT cohort, TSP and KM were scored by AR. For all microenvironment scoring, 10% of cases were co-scored in a blinded manner with an intra-class correlation co-efficient of >0.7.

These were combined as the GMS as previously described(Alexander et al., 2020c). In brief, strong KM and any TSP scored GMS 0; weak KM with low TSP scored GMS 1 and weak KM with high TSP scored GMS 2.

6.1.4 Statistical analysis

In the GRI-CRC-TMA, data were analysed using SPSS version 27.0 (IBM SPSS). For the TransSCOT cohort, data were analysed using SPSS version 25.0 (IBM SPSS) by Antonia Roseweir. Kaplan-Meier and log-rank analysis compared survival adjusted for T-stage, N-stage and treatment duration, where appropriate. Hazard ratios (HR) and confidence intervals (CI) were calculated from univariate Cox regression survival analysis. Multivariable survival analysis using a backward conditional elimination model and a statistical significance threshold of p-value<0.1 was performed to identify independent prognostic biomarkers. Text results are reported as HR, 95% CI for GMS 0 vs GMS 2, but p-value given is for log-rank analysis of overall trend. Pearson chi-squared test was used to test associations

between categorical variables and GMS. A Cox proportional hazard (PH) interaction model was performed to assess interactions between GMS and treatment type/duration. The study conformed to the REMARK guidelines(McShane et al., 2005) and statistical significance was set at p-value<0.05.

6.2 RESULTS

6.2.1 GMS and adjuvant chemotherapy in the GRI-CRC-TMA

The effect of chemotherapy vs no chemotherapy on CSS in high-risk disease (TNM III or Petersen index ≥2 in TNM II disease) according to GMS category was examined. It is noteworthy that of those with high-risk disease 187 patients did not receive chemotherapy and this would indicate a more comorbid group, which represents a bias in this analysis. Table 6.1 gives information on clinicopathological variables between the group receiving adjuvant chemotherapy vs none. Those who did not receive adjuvant therapy were older (p<0.001), had a poorer lymph node yield (p=0.03), were more systemically inflamed, according to mGPS (p=0.02), had a greater number of perforated tumours (p<0.05), trended towards higher T-stage (p=0.06) and, for those with information available, had a higher ASA grade (p<0.001). Table 6.2 provides details of analysis for each GMS category stratified by chemotherapy vs no chemotherapy. For GMS 0 (N=34), those receiving chemotherapy trended towards better survival (HR 0.14, 0.02-1.21, p=0.07, Figure 6.1A), although there were only 6 events in this subgroup, 5 of which were in the no chemotherapy group. For GMS 1 (N=223), those receiving chemotherapy had significantly better survival (HR 0.63, 0.40-0.97, p=0.04, Figure 6.1B). Finally, for GMS 2 (N=95), those receiving chemotherapy trended towards better survival (HR 0.57, 0.31-1.04, p=0.07, Figure 6.1C), but did not reach significance.

Table 6.1. Clinicopathological variables for patients receiving adjuvant chemo (*N*=167) vs none (*N*=187) in high-risk CRC

Clinicopathological characteristics			
	Chemo	No chemo	Chi square
	N (%)[a]	*N* (%)[a]	*P*
Age			
≤64	82 (49)	36 (19)	**<0.001**
65-74	62 (37)	61 (33)	
≥75	23 (14)	90 (48)	
Presentation			
Elective	150 (90)	158 (85)	0.14
Emergency	17 (10)	29 (15)	
Location			
Colon	118 (71)	134 (72)	0.84
Rectum	49 (29)	53 (28)	
TNM			
II (low-risk)	21 (13)	30 (16)	0.34
III (high-risk)	146 (87)	157 (84)	
T-stage			
T1	4 (2)	2 (1)	0.06
T2	8 (5)	9 (5)	
T3	91 (55)	85 (46)	
T4	64 (38)	91 (49)	
N-stage			
N0	21 (13)	30 (16)	0.20
N1	105 (63)	120 (64)	
N2	41 (26)	37 (20)	
Neoadjuvant therapy			
No	153 (94)	172 (92)	0.50
Yes	10 (6)	15 (8)	
Differentiation			
Well/mod	144 (87)	164 (88)	0.69
Poor	22 (13)	22 (12)	
Lymph node sample <12			
Sample 12 nodes +	135 (81)	132 (71)	**0.03**
Sample <12 nodes	32 (19)	55 (29)	
Peritoneal involvement			
Absent	107 (64)	104 (56)	0.11
Present	60 (36)	83 (44)	
Tumour perforation			
Absent	163 (98)	174 (93)	**0.046**
Present	4 (2)	13 (7)	
Venous invasion			
Absent	54 (32)	60 (32)	0.96
Present	113 (68)	127(68)	
mGPS			
0	115 (69)	109 (64)	**0.02**
1	35 (21)	43 (20)	
2	17 (10)	35 (19)	
ASA grade			
1	22 (21)	5 (4)	**<0.001**
2	47 (45)	52 (39)	
3	33 (32)	62 (47)	
4	2 (2)	13 (10)	

Table 6.2. Univariate CSS survival for adjuvant chemotherapy vs no chemotherapy in high-risk TNM according to GMS

Group		**Cancer-specific Survival**			
	N	10-year CSS (%; SE)	Events	HR (95% CI)	*P*
GMS 0					
No adjuvant chemotherapy	17	62 (14)	5	1.0 (reference)	
Adjuvant chemotherapy	17	92 (8)	1	0.14 (0.16-1.21)	0.07
GMS 1					
No adjuvant chemotherapy	119	51 (5)	48	1.0 (reference)	
Adjuvant chemotherapy	104	66 (5)	34	0.63 (0.40-0.97)	**0.04**
GMS 2					
No adjuvant chemotherapy	51	39 (8)	27	1.0 (reference)	
Adjuvant chemotherapy	44	59 (8)	17	0.57 (0.31-1.04)	0.07

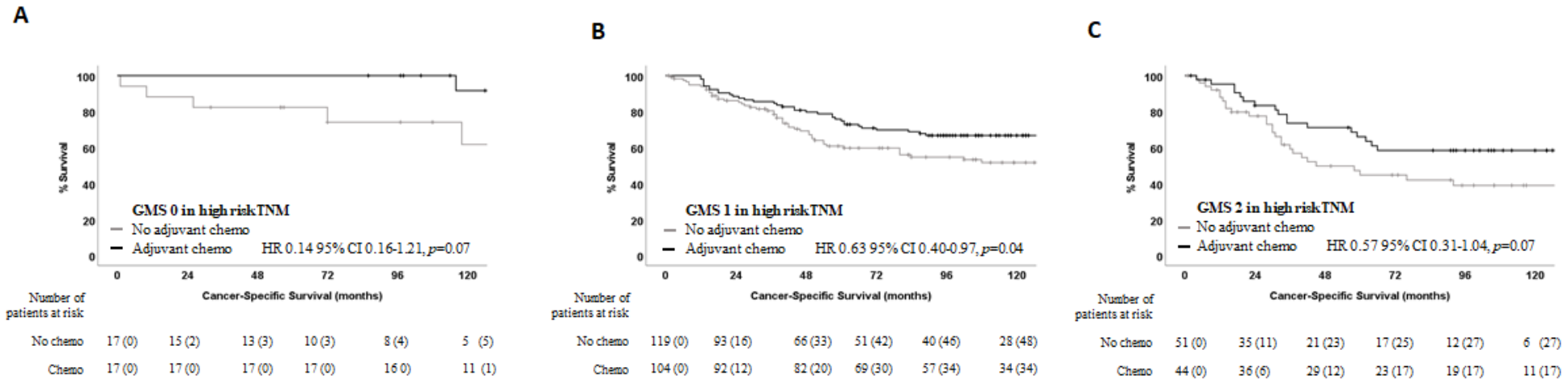


Figure 6.1. CSS according to administration of adjuvant chemotherapy stratified by GMS status. (A) GMS 0 (N=34); (B) GMS 1 (N=223); (C) GMS 2 (n=95)

6.2.2 Interactions between GMS and chemotherapy type/duration in the TransSCOT cohort

The interaction between GMS and adjuvant chemotherapy type and duration was investigated (Table 6.3). Multivariate Cox proportional hazard analysis was performed, demonstrating a significant interaction between GMS and chemotherapy type (p=0.01) but not duration (p=0.64). As an interaction was seen between GMS and chemotherapy type, associations with DFS where stratified for FOLFOX and CAPOX. For patients receiving FOLFOX, the association with DFS was strengthened with a 5-year DFS for GMS 0, 1 and 2 of 88%, 62% and 54%, respectively (GMS 0 vs GMS 2: HR 3.50 95% CI 1.88-6.50, p<0.001, Figure 6.2B). However, for patients receiving CAPOX these associations were dampened with a 5-year DFS for GMS 0, 1 and 2 of 62%, 63% and 53%, respectively (GMS 0 vs GMS 2: HR 1.33 95% CI 0.98-1.85, p=0.07, Figure 6.2C). As associations with DFS were strengthened in the FOLFOX-treated patients, patients were stratified by GMS category to assess if any group responded more favorably to one particular therapeutic regimen. Patients with GMS 0 significantly benefited from FOLFOX over CAPOX, with 5-year DFS of 88% vs 62% (HR 2.23 95% CI 1.19-4.16, p<0.001, Figure 6.2D). However, no difference in DFS was seen for GMS 1 with 5-year DFS for FOLFOX and CAPOX of 62% vs 63% (HR 1.08 95% CI 0.88-1.33, p=0.21, Figure 6.2E) or GMS 2 with 5-year of 54% vs 53%, respectively (HR 0.90 95% CI 0.68-1.19, p=0.68, Figure 6.2F). To ensure that the interaction between GMS 0 and chemotherapy type was not inadvertently due to one group receiving a longer course of chemotherapy than another, a further test of association was performed between type and duration of chemotherapy in the GMS 0 subgroup. There was no significant association between chemotherapy type and duration in this subgroup (p=0.11; Table 6.4).

Table 6.3. Interactions between GMS and chemotherapy Type or duration (*N*=2913).

Group		**GMS**						
	N	**0** *N* (%)[a]		**1** *N* (%)		**2** *N* (%)		Interaction *P*
Full cohort		383	–	1867	–	663	–	
Chemotherapy type								**0.013**
FOLFOX	846 (29)	120	(31)	526	(28)	200	(30)	
CAPOX	2067 (71)	263	(69)	1341	(72)	463	(70)	
Chemotherapy duration								0.64
3 months	1468 (50)	194	(51)	955	(51)	319	(48)	
6 months	1444 (50)	189	(49)	911	(49)	344	(52)	
Lower Risk Stage III Patients (T1-3/N1; n=1284)		202	–	861	–	221	–	
Chemotherapy type								**0.005**
FOLFOX	374 (29)	64	(32)	249	(29)	61	(28)	
CAPOX	910 (71)	138	(68)	612	(71)	160	(72)	
Chemotherapy duration								0.82
3 months	650 (51)	91	(45)	449	(52)	110	(50)	
6 months	634 (49)	111	(55)	412	(48)	111	(50)	
Higher Risk Stage III Patients (T4 and/or N2; n=1073)		102	–	643	–	327	–	
Chemotherapy type								0.61
FOLFOX	336 (31)	32	(31)	196	(31)	108	(33)	
CAPOX	736 (69)	70	(69)	447	(70)	219	(67)	
Chemotherapy duration								0.84
3 months	543 (29)	57	(56)	335	(52)	151	(46)	
6 months	529 (71)	45	(44)	308	(48)	176	(54)	

Table 6.4. Association between chemotherapy type and chemotherapy duration in GMS 0 subgroup (*N*=383).

	Chemotherapy type		**Pearson X^2**
	FOLFOX *N* (%)	**CAPOX** *N* (%)	
GMS 0 (n=383)			
Chemotherapy duration			*0.11*
3-months	68 (57)	52 (48)	
6-months	52 (43)	137 (52)	

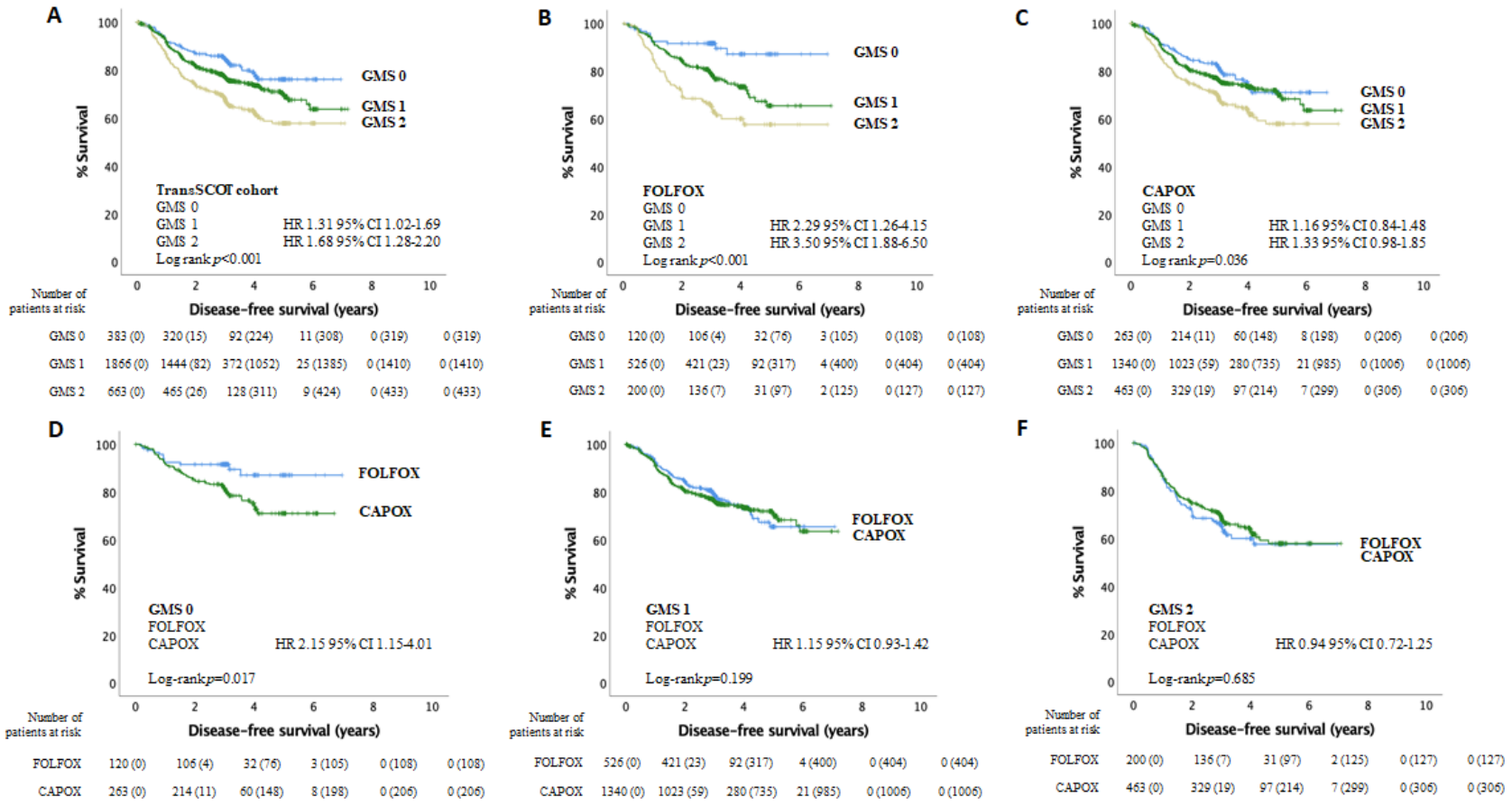


Figure 6.2. Disease-free survival according to GMS, stratified by chemotherapy type in TransSCOT cohort: (A) full TransScot cohort (n=2912); (B) FOLFOX patients (n=846) or (C) CAPOX patients (n=2066). (D-F) DFS according to chemotherapy type in: (D) GMS 0 (n=383), (E) GMS 1 (n=1866) or (F) GMS 2 (n=663)

Next, interactions with type and duration of chemotherapy were assessed (Table 6.3). GMS did not interact with duration in either group. GMS interacted with type of chemotherapy in lower-risk patients (p=0.005) but not higher-risk patients (p=0.61). For patients receiving FOLFOX, GMS stratified DFS in both the lower-risk (GMS 0 vs GMS 2: HR 5.41 95% CI 1.83-15.98, p=0.001, Figure 6.3C) and higher-risk disease (GMS 0 vs GMS 2: HR 2.61 95% CI 1.12-6.12, p=0.03, Figure 6.3D). However, when assessing chemotherapy type within TNM III patients with GMS 0, patients benefited from FOLFOX over CAPOX chemotherapy in lower-risk (HR 2.94 95% CI 1.02-8.47, p=0.04, Figure 6.3E), but not higher-risk disease (HR 1.82 95% CI 0.75-4.47, p=0.18, Figure 6.3F), although this was likely due to smaller numbers (n=102).

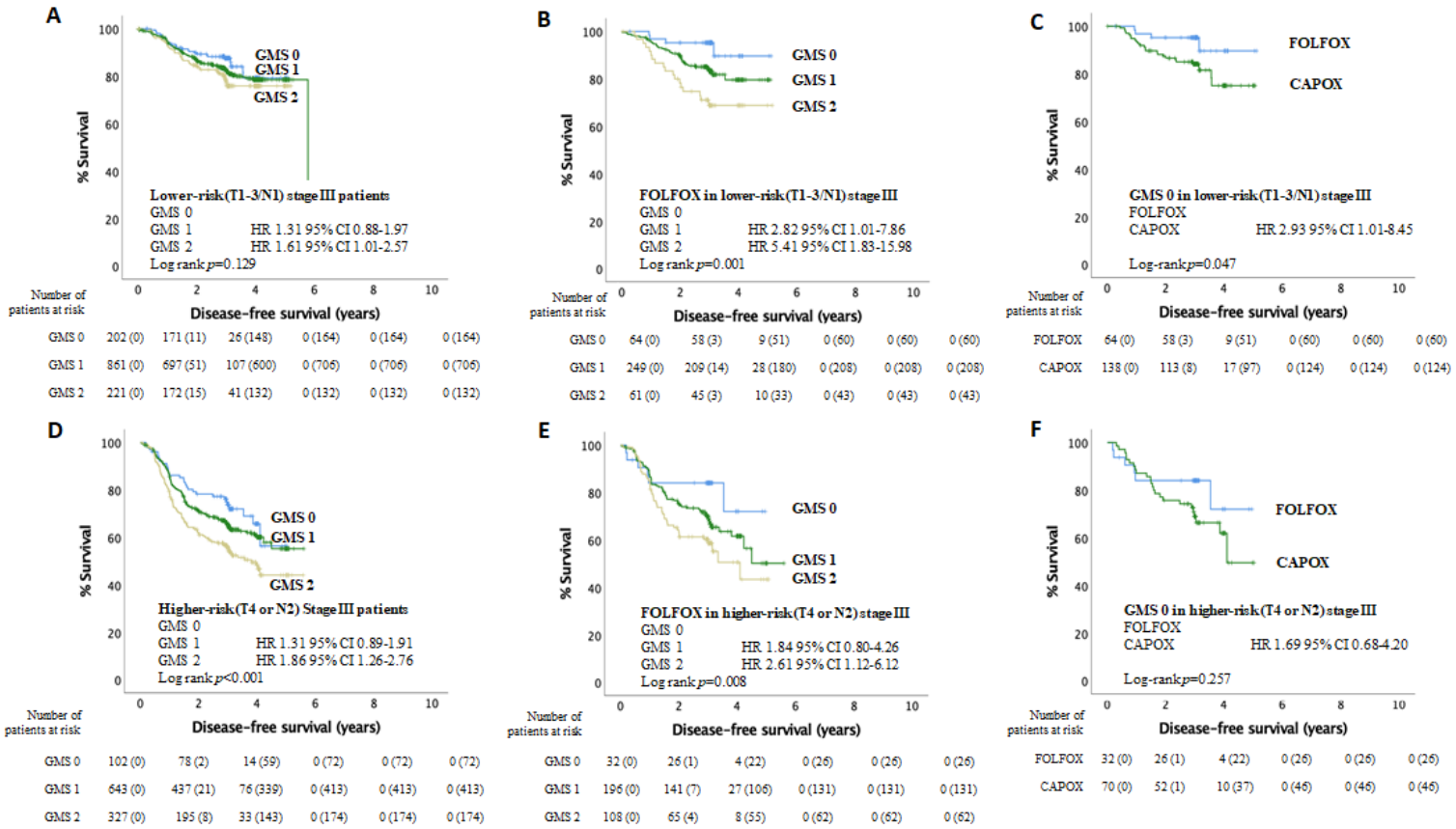


Figure 6.3. GMS, prognosis and response to adjuvant chemotherapy in lower-risk (A-C) and higher-risk (D-F) stage III patients from the TransSCOT cohort (*n*=2356). (A,D) Associations between GMS and DFS in (A) lower-risk (*n*=1284) and (D) higher-risk (*n*=1072) stage III patients. (B,E) Associations between GMS and DFS in (B) lower-risk (*n*=374) and (E) higher-risk (*n*=336) patients receiving FOLFOX adjuvant chemotherapy. (C,F) Associations between chemotherapy type and DFS in GMS 0 patients within the (C) lower-risk (*n*=202) and (F) higher-risk (*n*=102) stage III groups.

6.3 DISCUSSION

Given the high-risk nature of the GMS 2 phenotype, these tumours may warrant more aggressive follow up with an enhanced surveillance programme, in order to detect recurrent disease at an earlier stage. In these two cohorts, patients with GMS 2 tumours receiving adjuvant chemotherapy had a similarly poor prognosis. Previous work demonstrated that GMS 2 tumours do not respond well to conventional chemotherapy(Alexander et al., 2020c, Zunder et al., 2019). In the GRI-CTC-TMA cohort, those in the high-risk TNM group with GMS 2 who received chemotherapy did not have a superior survival to those not receiving chemotherapy, despite higher comorbidity for patients in the latter group. However, other novel agents may give more benefit to this subgroup. Zunder et al.(Zunder et al., 2018), reporting on the AVANT trial, stated that Bevacizumab, an anti-VEGF monoclonal antibody, in combination with FOLFOX-4 or XELOX showed a trend towards improved colon cancer survival vs standard chemotherapy alone, but only in the high stromal group (i.e. GMS 2).

In terms of limitations, the data for the GRI-CRC-TMA was collected outwith the rigorous follow up of a clinical trial and therefore, although data was taken from a prospectively maintained dataset, it is possible that patients were lost to follow up. Furthermore, the specific chemotherapeutic regimen used and full comorbidity data was not available, which limits analysis between those who received chemotherapy vs those who did not. Finally, a small number of patients received neo-adjuvant therapy for rectal cancer (n=54) and this is known to alter the appearance of the tumour microenvironment with the addition of fibrosis making assessment of TSP difficult(Hav et al., 2015). However, only 14 of these were deemed to have high TSP. Furthermore, GMS was independent of neoadjuvant therapy on multivariate analysis for survival.

The association of GMS with chemotherapy regimen was explored in the TransSCOT cohort. GMS survival stratification in the TransScot cohort was similar to that in the JP-AP TMA (see section 4.4 and 4.5). GMS 2 patients derived less benefit from adjuvant chemotherapy

independent of regimen used or risk stratification. GMS 1 patients did not respond better to any particular chemotherapy type but had an intermediate survival outcome. However, for GMS 0 subgroup, survival for patients receiving FOLFOX was significantly better than for those receiving CAPOX, especially in lower-risk TNM III. This did not appear to reflect differences in duration of chemotherapy.

Whilst further validation is required, the results suggest that those with strong peritumoural inflammation have different clinical outcomes depending on which form of 5-FU-based chemotherapy is administered. FOLFOX was shown to offer a more favourable outcome in the presence of strong peritumoural inflammation (GMS 0). However, in the absence of such an infiltrate (both GMS 1 and GMS 2), there was no survival difference.

Previous studies have reported that colorectal cancer patients receiving chemotherapy have better outcomes if they have higher tumour-infiltrating lymphocytes(Cha et al., 2019, Shibutani et al., 2018, Alexander et al., 2020b). However, there are no previously published studies that have compared the efficacy of FOLFOX vs CAPOX depending on peritumoural inflammation. The link between strong KM and type of chemotherapy was demonstrated by our group when investigating the 1343 TransScot patients studied for the Phenotypic Subtypes study(Roseweir et al., 2020). Since the assessment of Ki67 did not add to this differentiation, only the GMS was performed on the full TransScot cohort. There is, therefore, paucity of data as to the mechanism underlying this effect and further investigation is required. One hypothesis is that the elevated levels of immune cells hamper the final stage of capecitabine metabolism, inhibiting its cytotoxic effect and therefore dampening the effect of CAPOX. However, as previously stated, patients with strong peritumoural inflammation have better outcomes on adjuvant chemotherapy and so this explanation holds little weight. Alternatively, the administration of intravenous 5-FU in the FOLFOX regimen may result in better bioavailability of the active metabolite, fluoro-deoxyuridine monophosphate, than oral Capecitabine and this effect would be more pronounced in the

higher immune group. Further still, Folinic Acid (Leucovorin) is administered as part of the FOLFOX regimen as it has been found to enhance the anti-tumour effects of 5-FU(Priest et al., 1991). Folinic acid is an intravenous folate and is also used to supplement vitamin B9, which can protect against bone marrow suppression(Goldman and Matherly, 1987) and this may protect FOLFOX patients with strong peritumoural inflammation against the immunosuppressive side effects of chemotherapy. However, there are no studies to date exploring this phenomenon.

Pagès et al.(Pagès et al., 2020) recently published results comparing the Immunoscore in the French cohort of the IDEA study, finding that those with strong anti-tumour immunity might benefit from longer course mFOLFOX6. While the results of the TransScot cohort validate the use of FOLFOX over CAPOX in this patient group, there was no association between duration of treatment and GMS status.

A further limitation of the current study is the lack of overall and cancer-specific survival data in the TransScot cohort. However, as shown in the JP-AP TMA, the curves were very similar for DFS and overall survival and therefore, DFS can be considered a reasonable primary endpoint.

In conclusion, the present study validates the prognostic utility of the Glasgow Microenvironment Score. The poor outcome in low-risk disease of GMS 2 indicates that this subgroup may not derive benefit from standard adjuvant treatment. However, GMS 2 may be considered an additional high-risk feature that warrants consideration for novel therapies. Conversely, GMS 0 in high-risk patients highlights a sub-group that may benefit most from current therapies. This survival effect was strengthened in patients receiving FOLFOX but dampened in patients receiving CAPOX. Therefore, GMS could be a useful tool to aid both prognostic and therapeutic decision making in clinical practice alongside TNM-staging. GMS should be further assessed in the context of prospective randomised clinical trials.

7. Programmed-Cell Death 1 (PDCD1) and Programmed Death Ligand-1 (CD274)

Finally, the role of Programmed-Cell Death 1 (PDCD1) and Programmed Death Ligand-1 (CD274) in CRC has not yet been determined. To this end, the following literature review and meta-analysis was performed in order to clarify both the prognostic role of PDCD1 and CD274 assessed in different cellular domains on pathological slides and also in the context of the predictive role for CD274 with regard to response to chemotherapeutic agents as far as clinical trials have reported its use.

Programmed cell death protein-1 (PDCD1 or PD-1) is a cell surface protein initially discovered by Honjo et al. in the 1990s(Freeman et al., 2000). Expression of the PDCD1 ligand, programmed death ligand-1 (CD274 or PD-L1), on tumour and antigen presenting cells can cause down-regulation of the adaptive anti-tumour immune response, but monoclonal antibody-mediated inhibition of this interaction facilitates a re-invigorated immune response(Iwai et al., 2005). More recently, CD274 expression on antigen-presenting immune cells has been shown, by multiplex-fluorescent immunohistochemistry to reduce cytotoxic T-cell and tumour cell interaction(Lazarus et al., 2019).

Although it may be assumed that high CD274 expression is a marker of poorer prognosis in patients with CRC, published literature to date has been limited not only by wide variability in reported immunohistochemical techniques and scoring methodologies, but also in the incongruity of which cell populations within the microenvironment were assessed (i.e. tumour or immune).

The tumour percentage score (TPS), a measure of the proportion of strong-staining CD274 tumour cells to total tumours cells, has been proposed as a measure of CD274 activity in patients with lung cancer. However, in gastro-oesophageal cancer, this was not found to accurately identify those who will respond to immune checkpoint inhibitors(Martin and Markl, 2019). Therefore, the combined positive score (CPS) was developed, which is

calculated by dividing the total cells above the threshold for CD274 positivity (both tumour and immune) by the total number of viable tumour cells. This was found to be an effective measure of response to anti-PD-1 therapy, particularly when using a higher threshold (>10)(Wainberg et al., 2020).

However, there is currently no standardised method of measuring PDCD1 or CD274 in CRC. Furthermore, microsatellite instability (MSI) may play a pivotal role in CRC response to immune checkpoint inhibition, with several trials reporting therapeutic benefit to immune checkpoint inhibitors in only those with MSI tumours(Le et al., 2015). This therefore represents a significant confounder in any published literature that should be taken into account in multivariate analysis.

Despite this, a number of ongoing clinical trials are investigating the potential of anti-PD-1 therapy in patients with MSS CRC. It is known that high immune MSS cancers also have an improved survival(Alexander et al., 2020b). Those who relapse in this group are likely to be developing similar immune escape pathways to MSI tumours. Therefore, a CD274 score that can correctly identify patients who will respond to anti-PD-1 therapy is required moving forward in CRC.

The aims of this study are two-fold. Firstly, to perform a meta-analysis of the prognostic significance of PDCD1/CD274 in patients with CRC and secondly, to review the current anti-PD-1 therapy trial results, with particular reference to those assessing response in the light of CD274 status.

7.1 SPECIFIC METHODS

A brief description of methods has already been made in the Generic Methods section. Here follows a more detailed description of certain aspects of the literature review.

7.1.1 Search Strategy

The following search terms were entered into PubMed, Ovid, MEDLINE and EMBASE databases:

- "colorectal cancer" or "colon cancer" or "rectal cancer" (Abstract) AND
- "prognos$" or "survival" (Abstract) AND
- "immunohistochemistry" (any field) AND

• "PD1" or "PD-1" or "PDCD1" or "CD279" or "programmed cell death" (Abstract)	**OR**	• "PDL1" or "PD-L1" or "B7-H1" or "CD274" (Abstract)"

The search was limited to English language articles, published after 1997 and human studies. Search was performed by PGA and all primary studies were identified via abstracts +- review of the main article for data extraction. A search was also made of the reference sections of the articles identified to assess if any studies had been overlooked in the literature search. Inclusion criteria included: prospective or retrospective design, but a well-defined study population; primary cancer resections for colon, rectal or colorectal cancer; use of FFPE slides and IHC staining for PDCD1 or CD274; clear description in the methods of the specimen, antibodies and counting method employed and tumour compartment assessed; description of groups assessed and thresholds employed; statistical analysis method; and, for the purposes of meta-analysis, inclusion of a proportional hazards model with details of any adjustment methods. Studies utilizing only multiplex-fluorescent IHC were excluded. Any contentious articles were discussed with DCM or JHP to agree those for inclusion.

In addition to the above search, current clinical trials including anti-PD-1 therapy (Nivolumab/Pembrolizumab/Spartalizumab/Durvalumab/Atezolizumab/Amp-224/Avelumab/BAT1306/Tislelizumab/Cetrelimab/Camrelizumab/Toripalimab/Cosibelimab/M7824/Sintilimab/Genolizumab/MGA012/BI754091/Zimberelimab/Dostarlimab/XmAb20717[dual PD-1 and CTLA4 inhibitor]) in colorectal cancer (registered at Clinicaltrials.gov or clinicaltrialsregister.eu) were reviewed, along with any published results or relevant conference abstracts displaying interim results.

7.1.2 Data Extraction

Generic data on the year of study and clinicopathological data on the individual patient population(s) were extracted. Further important data extracted included type of specimen studied, whether tissue microarray (TMA, and size of core, if given) or full resection specimen, method of PCDC1/CD274, which immunohistochemical stain was used, MSI status of tumours and treatment of this in the statistical analysis, which survival outcome was used (cancer-specific, overall, disease-free, etc.) and Hazard ratios/*p*-values if provided. All relevant studies were included in literature review, but only studies that performed multivariate analysis with hazard ratios were included in meta-analysis. Figure 7.1 shows the flow diagram of studies used in systematic review and meta-analysis.

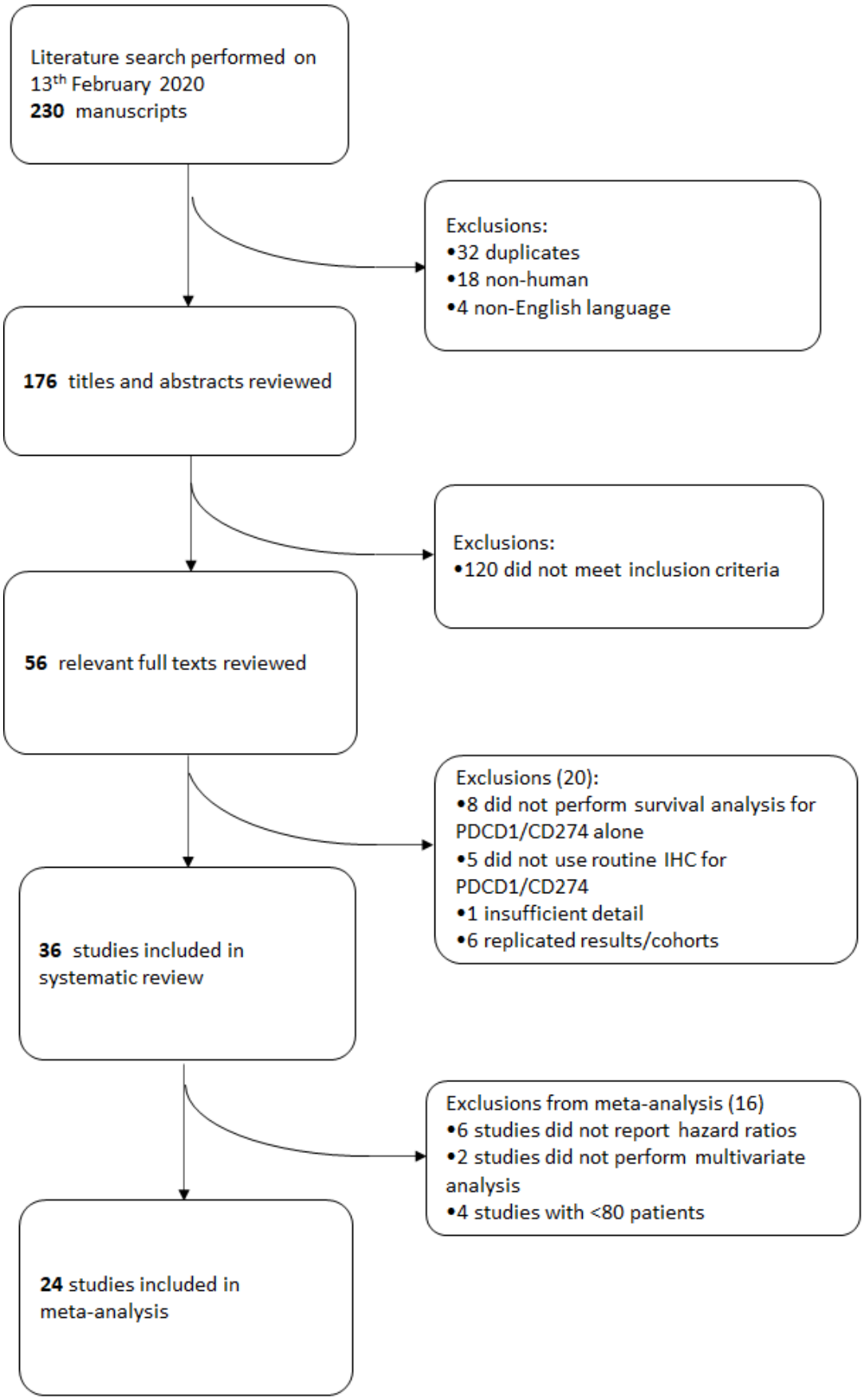


Figure 7.1. Flow diagram indicating reasons for excluding studies from systematic review and meta-analysis

7.1.3 Statistical analysis

For all inflammatory assessments, studies were grouped according to tumour location i.e. colon, rectal or colorectal. Furthermore, studies were collated according to the specific method of survival analysis: disease-free survival (DFS) was defined as the length of time between diagnosis and recurrence or spread of disease ("recurrence-free survival" and "progression-free survival" were also grouped under this heading); overall survival (OS) was defined as the length of time between diagnosis and death from any cause; disease-specific survival (DSS) was defined as the length of time between diagnosis and death from CRC-related cause ("cancer-specific survival" was also included under this heading). Furthermore, differentiation was made between PDCD1 or CD274 assessed on tumour cells or assessed on inflammatory cells. Studies of small sample size (n<80) were excluded from meta-analysis due to potential for bias. REVMAN (version 5.3) software was used for meta-analysis. Funnel plots were used to assess the potential for publication bias, while forest plots and I^2 values were used to assess inter-study heterogeneity. Multiple studies in the same category are presented with a fixed effects summary HR and 95% CI, whereas in the event of only a single study being included in a particular category, the HR and 95% CI are given for that study. Confidence intervals were considered not significant where they crossed 1.0.

7.2 OVERVIEW OF STUDIES

7.2.1 Search results and exclusions

The initial literature search (see Figure 7.1) yielded 230 results, which was reduced to 176 after limiting to English language (*n*=4), human studies (*n*=18) and following deduplication (*n*=32). Abstracts were reviewed and a further 120 were excluded as they did not meet inclusion criteria. Full texts were obtained for the remaining 56 relevant texts and, after careful scrutiny, 20 more were excluded for lack of survival analysis (*n*=8), non-standard IHC (*n*=5), replicated results/cohort (*n*=6) or insufficient detail (*n*=1), leaving 36 studies. Finally, for meta-analysis, a further 12 studies had to be excluded for lack of hazard ratios (*n*=6), no multivariate analysis (*n*=2) and cohort size smaller than 80 subjects (*n*=4). The final 24 studies were included in meta-analysis, with methodologies summarised in Appendix 2, Table A2.1. Bias assessment for the included studies is shown in Table 7.1, all of which were considered low-risk for bias (the only study considered moderate risk had already been excluded for insufficient detail).

Table 7.1. Assessment of bias of studies included in meta-analysis[a]

First author, year (ref)	Centre	Population well defined (selection bias)	Method of assessment specified (selection bias)	Threshold defined (selection bias)	Group allocation defined (observer bias)	Blinding (observer bias)	Evaluation by >1 observer (observer bias)	Loss to follow up (attrition bias)	Patient and tumour characteristics (reporting bias)	Follow up defined/ Specified (reporting bias)	Risk of bias [a]	Bias High/ med/ low
Bae 2018 (Bae et al., 2018)	DMC, South Korea	1	**0**	**1**	**1**	**0**	**0**	**1**	**1**	**1**	**6**	**Low**
Berntsson 2018 (Berntsson et al., 2018)	EPIC Study, Sweden	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**9**	**Low**
Calik 2019 (Calik et al., 2019)	FUH, Turkey	1	**1**	**1**	**1**	**0**	**1**	**1**	**1**	**1**	**8**	**Low**
Chen 2019 (Chen et al., 2019b)	CMUH, Taiwan	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0**	**8**	**Low**
D'Alterio 2016 (D'Alterio et al., 2016)	Napoli, Italy	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**9**	**Low**
Droeser 2013 (Droeser et al., 2013)	UoB, Switzerland	1	**0**	**1**	**1**	**1**	**1**	**1**	**1**	**0**	**7**	**Low**
Enkhbat 2018 (Enkhbat et al., 2018)	TUH, Japan	1	**1**	**1**	**1**	**1**	**0**	**1**	**1**	**1**	**8**	**Low**
Eriksen 2019 (Eriksen et al., 2019)	DCCG, Denmark	1	**1**	**1**	**1**	**0**	**1**	**1**	**1**	**1**	**8**	**Low**
Hamada 2017 (Hamada et al., 2017)	NHS+HPFS studies, USA	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**9**	**Low**
Hecht 2016 (Hecht et al., 2016)	UHE, Germany	1	**0**	**1**	**1**	**1**	**0**	**1**	**1**	**1**	**7**	**Low**

Ho 2019 (Ho et al., 2019)	TVGH, Taiwan	0	**1**	**1**	**1**	**1**	**0**	**1**	**1**	**0**	**6**	**Low**
Huang 2018 (Huang et al., 2018)	CMUH, Taiwan	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0**	**8**	**Low**
Koganemaru 2017 (Koganemaru et al., 2017)	TH, Japan	1	**1**	**1**	**1**	**0**	**1**	**1**	**1**	**1**	**8**	**Low**
Ledys 2018 (Ledys et al., 2018)	CGFL, France	0	**1**	**1**	**1**	**0**	**1**	**1**	**1**	**1**	**7**	**Low**
Lee 2016 (Lee et al., 2016)	MSK, USA	0	**1**	**1**	**1**	**0**	**0**	**1**	**1**	**1**	**6**	**Low**
Lee 2017 (Lee et al., 2017c)	SNUBH, South Korea	1	**1**	**1**	**1**	**0**	**1**	**1**	**1**	**0**	**7**	**Low**
Lee 2018 (Lee et al., 2018b)	KNUCH, South Korea	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**9**	**Low**
Lee 2018[b] (Lee et al., 2018a)	SNUBH, South Korea	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**9**	**Low**
Li 2016 (Li et al., 2016)	FUSCC, China	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**9**	**Low**
Liu 2018 (Liu et al., 2018)	ZH, China	1	**1**	**1**	**1**	**0**	**0**	**1**	**1**	**0**	**6**	**Low**
Miller 2017 (Miller et al., 2017)	SJoGSH, W. Australia	1	**1**	**1**	**1**	**1**	**0**	**1**	**1**	**1**	**8**	**Low**
Rosenbaum 2016 (Rosenbaum et al., 2016)	MGH, USA	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0**	**8**	**Low**
Saigusa 2016 (Saigusa et al., 2016)	MUH, Japan	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**9**	**Low**
Shao 2017 (Shao et al., 2017)	FPCH, China	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**9**	**Low**

Shi 2013 (Shi et al., 2013)	FAH, China	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**9**	**Low**
Wei 2018 (Wei et al., 2018)	SYSUCC, China	1	**1**	**1**	**1**	**1**	**0**	**1**	**1**	**1**	**8**	**Low**
Wu 2019 (Wu et al., 2019)	AHXMU, China	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**9**	**Low**
Zhu 2015 (Zhu et al., 2015)	TH, China	1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**9**	**Low**

[a]Assessment of bias table score developed from REMARK guidelines[23], total out of 9: scores of 0-3 were considered high-risk for bias; scores of 4 or 5, moderate; and scores of 6 and above, low-risk.

[b]18 MSIH patients crossover, results given for different antibody

7.2.2 Study characteristics

Of the studies included in the meta-analysis, 11 performed assessment on TMAs, 10 on whole sections, 1 on pre-treatment biopsies and 2 on a combination of pre-treatment biopsy and post-resection TMA. TMA core sizes varied between 0.6mm and 3mm, with only 1 study not stating size of core. Eighteen studies documented blinding of assessors, whereas 8 studies did not comment on blinding. Sample size varied between 89 and 1105, with four studies having a sample size >500. Median sample size was 190 with an interquartile range of 117 to 338. Fifty percent of studies assessed MSI, although 2 of these studies did not include MSI in the survival analysis. There were over 25 variables included in the different multivariate analyses, of which the most common were age (*n*=19), sex (*n*=16), tumour grade (*n*=16), T-stage (*n*=14), N-stage (*n*=13), other inflammatory assessment (*n*=13), venous/lymphatic/peri-neural invasion (*n*=11), tumour site (*n*=10), TNM (*n*=9), M-stage (*n*=7) and MSI (*n*=5).

There was also a wide variation in assessment methods. Two studies only assessed CD274 on immune cells, whereas 9 studied CD274 only on tumour tissue. Four studies performed a combined assessment of CD274 on tumour tissue and immune cells, whereas 5 assessed CD274 on tumour tissue and immune cells separately. Others included assessment of PDCD1, with 1 study only assessing PDCD1 on immune cells, another assessing CD274 on tumour tissue and PDCD1 on immune cells, another CD274 on tumour tissue, but both CD274 and PDCD1 on immune cells and another combined tumour and immune cell CD274 assessment and separate PDCD1 assessment on immune cells.

Studies also differed on whether they assessed membranous staining (*n*=9), cytoplasmic staining (*n*=2), combined membrane and cytoplasmic staining (*n*=1) or any staining (*n*=9). Finally the threshold used by each study differed, with tumour tissue thresholds of 1% (*n*=3), 5% (*n*=7), 50% (*n*=2), semiquantitative assessment (*n*=4), immunoreactivity score or weighted histoscore (*n*=3), or arbitrary threshold, such as median (*n*=2). Immune cell

thresholds were 1% (*n*=2), 5% (*n*=5), 10% (*n*=1), 20% (*n*=1), >50% (*n*=1), semiquantitative (*n*=2), immunoreactivity score (*n*=1) or arbitrary (*n*=2).

7.3 SYSTEMATIC REVIEW AND META-ANALYSIS RESULTS FOR PDCD1 AND CD274 ASSESSMENT ACCORDING TO METHODOLOGY

7.3.1 PDCD1 (PD-1) assessment in immune cells

Immune cell expression of PDCD1 was assessed in 11 studies(Berntsson et al., 2018, Droeser et al., 2013, Huang et al., 2018, Kollmann et al., 2017, Lee et al., 2016, Li et al., 2016, Enkhbat et al., 2018, D'Alterio et al., 2016, Lee et al., 2018b, Shibutani et al., 2017b, Wei et al., 2018), comprising a total of 2498 patients. In 6 of these studies (1466 patients) PDCD1 immune cell expression was found to have a statistically significant beneficial survival impact(Berntsson et al., 2018, Droeser et al., 2013, Huang et al., 2018, Kollmann et al., 2017, Lee et al., 2016, Li et al., 2016). One study of 116 patients found immune PDCD1 expression to have a significant detrimental survival impact(Enkhbat et al., 2018), whereas four studies (595 patients) found no impact on survival(D'Alterio et al., 2016, Lee et al., 2018b, Shibutani et al., 2017b, Wei et al., 2018).

Six studies assessed for the presence of MSI, of which: two found that MSI was not significant for survival(Li et al., 2016, Wei et al., 2018); one study only included MSI tumours(Lee et al., 2018b) and they and one other study(Li et al., 2016) found PDCD1 not to be significant for survival in MSI patients; another study found that PDCD1 was only significant for survival (beneficial) in MSI patients(Lee et al., 2016); two studies found that PDCD1 was only significant for survival (beneficial) in MSS patients(Droeser et al., 2013, Li et al., 2016); and one study found that survival according to PDCD1 expression was not independent of MSI status in multivariate analysis(Berntsson et al., 2018).

Five studies, with 6 cohorts of patients met inclusion criteria for meta-analysis (Table 7.2, Figure 7.2): three assessed DFS (HR 0.50; 95% CI: 0.34-0.73), with no significant

heterogeneity; four cohorts were studied assessing OS (HR 0.74; 95% CI: 0.60-0.89), with significant heterogeneity; and all-cause survival (HR 0.72; 95% CI: 0.59-0.87) was significantly heterogeneous. Funnel plots did not suggest any significant publication bias, although numbers of studies were small (Figure 7.2).

Table 7.2. Meta-analysis results for survival in colorectal cancer according to PDCD1/CD274 expression on immune and tumour cells

		Overall effect			Heterogeneity		
Colonic site	**Survival type**	**No. of studies**	**HR**	**95% CI**	**I^2 test (%)**	***P*-value**	**First Author Surname/year**
PDCD1 high immune cells							
R	DFS	1	0.22	0.05-0.94	NA		Huang
CR	DFS	2	0.53	0.36-0.78	0	0.65	Wei, Li 16 (FUSCC)
Any	DFS	3	0.50	0.34-0.73	0	0.47	Huang, Wei, Li 16 (FUSCC)
CR	OS	5	0.74	0.60-0.89	74	0.004	Enkhbat, Wei, Li 16 (FUSCC), Li 16 (TCGA), Berntsson
Any	Any	6	0.72	0.59-0.87	72	0.003	Huang, Enkhbat, Wei, Li 16 (FUSCC), Li 16 (TCGA), Berntsson
CD274 high immune cells							
CR	DFS	4	0.43	0.31-0.60	67	0.03	Calik, Koganemaru, *Ledys*, **Lee 18 (Kyungpook)**
CR	OS	5	0.50	0.43-0.59	49	0.10	Berntsson, Ho, **Lee 17 (Bundang; MSIH)**, Lee 17 (Bundang; MSS), Lee 18 (Bundang)
CR	Any	9	0.49	0.42-0.57	54	0.03	Calik, Koganemaru, *Ledys*, **Lee 18 (Kyungpook)**, Berntsson, Ho, **Lee 17 (Bundang; MSIH)**, Lee 17 (Bundang; MSS), Lee 18 (Bundang)
CD274 high tumour cells							
R	DFS	1	0.34	0.16-0.72	NA		Chen
C	DFS	1	1.43	0.77-2.66	NA		Eriksen
CR	DFS	5	1.20	0.93-1.57	84	<0.001	Calik, Enkhbat, Koganemaru, *Ledys*, Li 16 (FUSCC)
Any	DFS	7	1.10	0.87-1.38	84	<0.001	Chen, Eriksen, Calik, Enkhbat, Koganemaru, *Ledys*, Li 16 (FUSCC)
CR	CSS	3	1.85	1.19-2.88	24	0.27	Hamada, Rosenbaum, Saigusa
R	OS	1	0.15	0.05-0.47	NA		Chen
C	OS	1	1.10	0.60-2.02	NA		Eriksen
CR	OS	13	0.87	0.84-0.91	83	<0.001	Berntsson, Droeser, Enkhbat, Hamada, Ho, *Ledys*, Lee 18 (Bundang), Li 16 (FUSCC), Li 16 (TCGA), Saigusa, Shi, Wu, Zhu
Any	OS	15	0.87	0.83-0.91	83	<0.001	Chen, Eriksen, Berntsson, Droeser, Enkhbat, Hamada, Ho, *Ledys*, Lee 18 (Bundang), Li 16 (FUSCC), Li 16 (TCGA), Saigusa, Shi, Wu, Zhu
Any	Any	18	0.88	0.84-0.92	84	<0.001	Chen, Eriksen, Calik, Koganemaru, Berntsson, Droeser, Enkhbat, Hamada, Ho, *Ledys*, Lee 18 (Bundang), Li 16 (FUSCC), Li 16 (TCGA), Saigusa, Shi, Wu, Zhu, Rosenbaum
CD274 high combined tumour and immune cells							
CR	DFS	2	0.98	0.71-1.34	94	<0.001	Bae, Wei
C	CSS	1	0.54	0.23-1.28	NA		Miller
R	OS	1	0.34	0.14-0.82	NA		Hecht

C	OS	1	1.00	0.38-2.66	NA		Miller
CR	OS	2	1.06	0.86-1.30	80	0.002	Bae, Wei
Any	OS/Any	4	1.00	0.82-1.21	80	0.002	Hecht, Miller, Bae, Wei

Bold studies: MSIH only; *Italics* studies: stage IV only.

Abbreviations: R rectal; C colon; CR colorectal

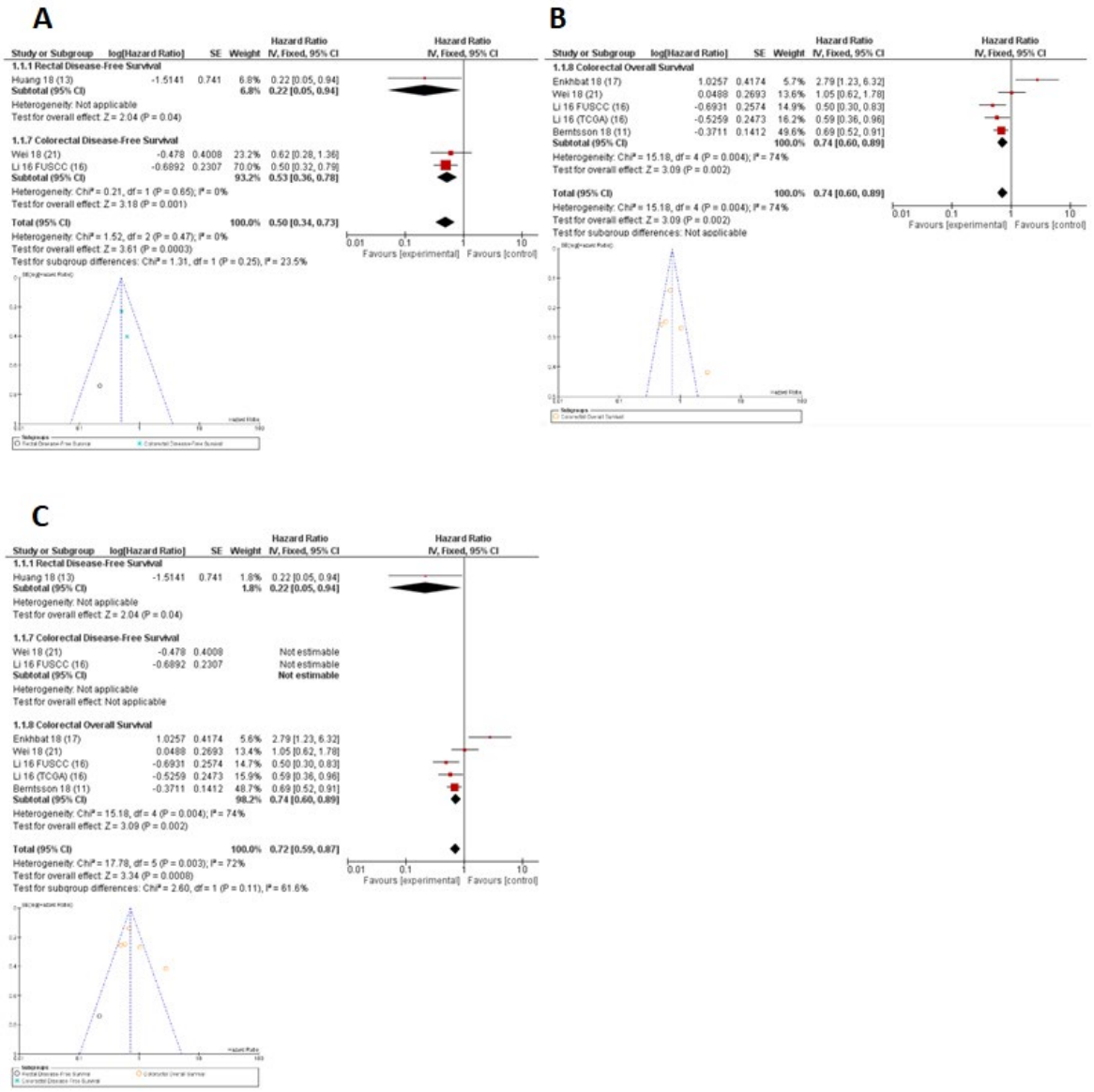


Figure 7.2. Forest plots and funnel plots for PDCD1 expression on Immune cells for A) DFS, B) OS and C) All survival

7.3.2 CD274 (PD-L1) assessment in immune cells

Immune cell expression of CD274 was assessed in 19 studies(Berntsson et al., 2018, Calik et al., 2019, Hecht et al., 2016, Ho et al., 2019, Koganemaru et al., 2017, Kollmann et al., 2017, Lee et al., 2017c, Lee et al., 2018b, Lee et al., 2018a, Li et al., 2016, Wyss et al., 2019, D'Alterio et al., 2016, Liu et al., 2018, Kim et al., 2016, Ledys et al., 2018, Ogura et al., 2018, Shao et al., 2017, Yomoda et al., 2019, Zhang et al., 2019), comprising a total of 3729 patients. One study(Lee et al., 2018a) must be presumed to have an overlap of 18 MSI patients with another in the same centre(Lee et al., 2017c). The dates only overlapped for the MSI cohort in this study(Lee et al., 2018a). In 11 studies, comprising 2718 patients, CD274 immune cell expression was found to have a beneficial survival impact(Berntsson et al., 2018, Calik et al., 2019, Hecht et al., 2016, Ho et al., 2019, Koganemaru et al., 2017, Kollmann et al., 2017, Lee et al., 2017c, Lee et al., 2018b, Lee et al., 2018a, Li et al., 2016, Wyss et al., 2019). Two small studies, comprising 93 patients, found immune cell CD274 expression to have a detrimental survival impact(D'Alterio et al., 2016, Liu et al., 2018), both of which assessed only stage IV disease, whereas of the other two studies assessing stage IV disease, one found a beneficial survival impact(Kollmann et al., 2017) and one found no survival impact(Ledys et al., 2018). Six studies of 918 patients found no survival impact(Kim et al., 2016, Ledys et al., 2018, Ogura et al., 2018, Shao et al., 2017, Yomoda et al., 2019, Zhang et al., 2019).

Eight studies assessed for the presence of MSI, of which: one found that MSI was not significant for survival(Lee et al., 2018a); one did not include MSI in survival analysis(Lee et al., 2018a); two reported immune CD274 to be independent of MSI(Berntsson et al., 2018, Wyss et al., 2019); four presented results for MSI cohorts of which two were significant for survival (beneficial),(Lee et al., 2017c, Lee et al., 2018b), whereas two were not significant(Kim et al., 2016, Li et al., 2016); two presented MSS cohorts, both of which were significant for survival (beneficial),(Li et al., 2016, Lee et al., 2017c).

Eight studies, with 9 cohorts met inclusion criteria for meta-analysis (Table 7.2, Figure 7.3): four assessed DFS (HR 0.43; 95% CI: 0.31-0.60), with moderate heterogeneity; five cohorts were studied assessing OS (HR 0.50; 95% CI: 0.43-0.59), with mild heterogeneity; and all-cause survival (HR 0.49; 95% CI: 0.42-0.57) was moderately heterogeneous. Funnel plots suggested possible publication bias against smaller, non-significant studies (Figure 7.3).

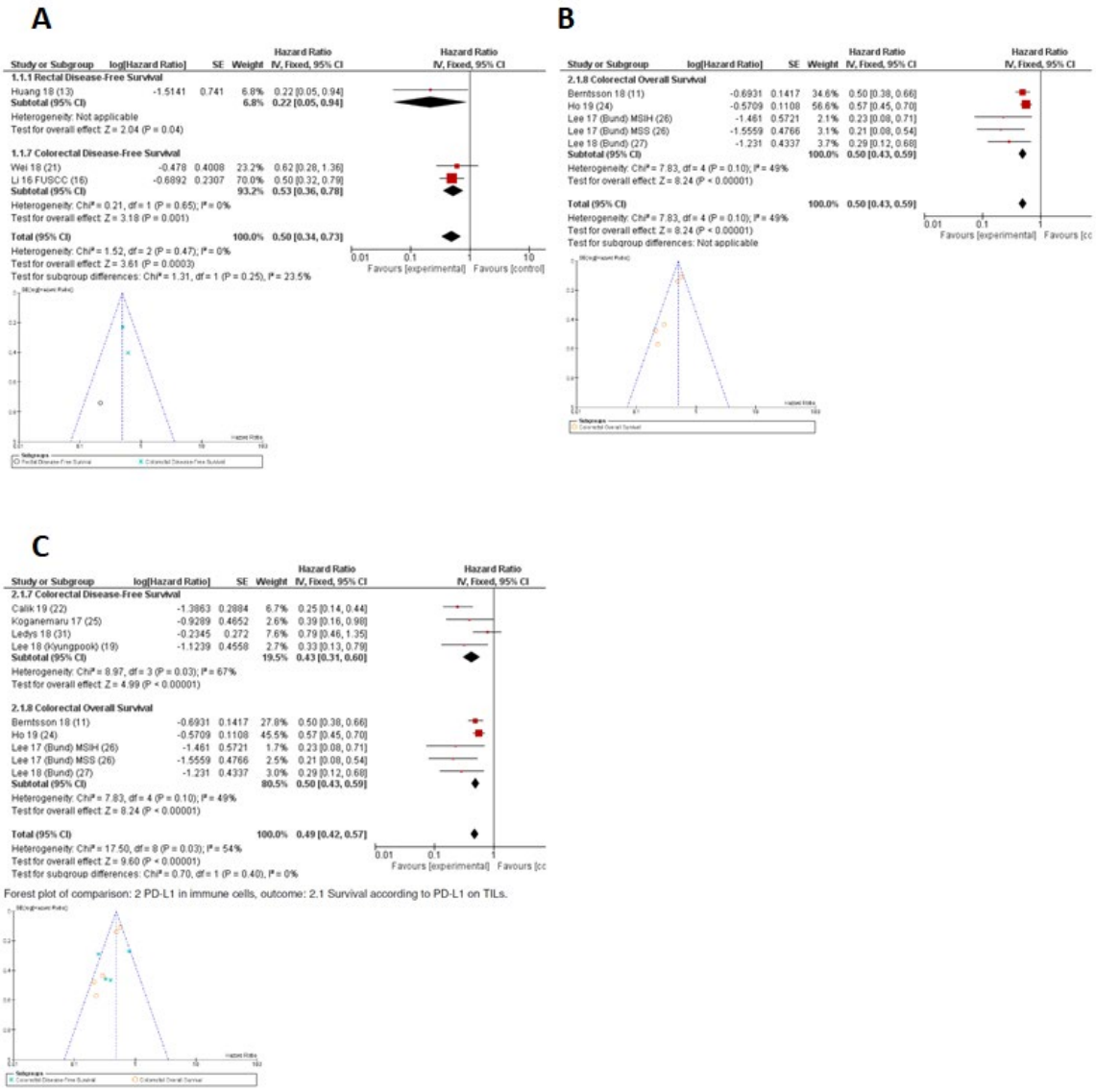


Figure 7.3. Forest plots and funnel plots for CD274 (PD-L1) expression on Immune cells for A) DFS, B) OS and C) All survival

7.3.3 CD274 (PD-L1) assessment in tumour tissue

Tumour tissue expression of CD274 was assessed in twenty-eight studies(Chen et al., 2019b, Droeser et al., 2013, Li et al., 2016, Shi et al., 2013, Calik et al., 2019, Chen et al., 2020, Enkhbat et al., 2018, Koganemaru et al., 2017, Lee et al., 2016, Lee et al., 2018a, Saigusa et al., 2016, Wu et al., 2019, Zhu et al., 2015, Berntsson et al., 2018, D'Alterio et al., 2016, Eriksen et al., 2019, Hamada et al., 2017, Hecht et al., 2016, Ho et al., 2019, Kim et al., 2016, Kollmann et al., 2017, Ledys et al., 2018, Lee et al., 2017c, Lee et al., 2018b, Ogura et al., 2018, Rosenbaum et al., 2016, Shao et al., 2017, Wyss et al., 2019), comprising 7054 patients. One study(Lee et al., 2018a) must be presumed to have an overlap of 18 MSI patients with another in the same centre(Lee et al., 2017c). The dates only overlapped for the MSI cohort in this study(Lee et al., 2018a). In 4 studies, comprising 1636 patients, expression of CD274 on tumour tissue was found to have a significant (beneficial) impact(Chen et al., 2019b, Droeser et al., 2013, Li et al., 2016, Shi et al., 2013). Whereas nine studies, comprising 1461 patients, found CD274 in tumour tissue to be associated with significant detrimental survival impact(Calik et al., 2019, Chen et al., 2020, Enkhbat et al., 2018, Koganemaru et al., 2017, Lee et al., 2016, Lee et al., 2018a, Saigusa et al., 2016, Wu et al., 2019, Zhu et al., 2015). Fifteen studies (3636 patients) assessing tumour tissue CD274 expression did not find any significant survival impact(Berntsson et al., 2018, D'Alterio et al., 2016, Eriksen et al., 2019, Hamada et al., 2017, Hecht et al., 2016, Ho et al., 2019, Kim et al., 2016, Kollmann et al., 2017, Ledys et al., 2018, Lee et al., 2017c, Lee et al., 2018b, Ogura et al., 2018, Rosenbaum et al., 2016, Shao et al., 2017, Wyss et al., 2019).

Fourteen studies assessed for the presence of MSI, of which tumour tissue CD274 was not significant for survival in nine(Berntsson et al., 2018, Eriksen et al., 2019, Hamada et al., 2017, Ho et al., 2019, Kim et al., 2016, Lee et al., 2017c, Lee et al., 2018b, Rosenbaum et al., 2016, Wyss et al., 2019), three of these assessing MSI only cohorts(Kim et al., 2016, Lee et al., 2017c, Lee et al., 2018b), although MSI was associated with higher expression of

CD274(Rosenbaum et al., 2016). Of the other 5 studies, only 2 patients had MSI in one(Chen et al., 2019b), two found that CD274 was associated with survival (beneficial) in the MSS subgroup(Droeser et al., 2013, Li et al., 2016), one found CD274 had a significant association with survival (detrimental) in the MSI subgroup(Lee et al., 2016) and one did not include MSI in survival analysis(Lee et al., 2018a).

Seventeen studies, with eighteen cohorts met inclusion criteria for meta-analysis (Table 7.2, Figure 7.4): seven assessed DFS (HR 1.10; 95% CI: 0.87-1.38), with significant heterogeneity; three studies assessed CSS (HR 1.85; 95% CI: 1.19-2.88), with no significant heterogeneity; 15 cohorts were studied assessing OS (HR 0.87; 95% CI: 0.83-0.91), with significant heterogeneity; and all-cause survival (HR 0.88; 95% CI: 0.84-0.92) was significantly heterogeneous. Funnel plot analysis did not suggest any publication bias (Figure 7.4).

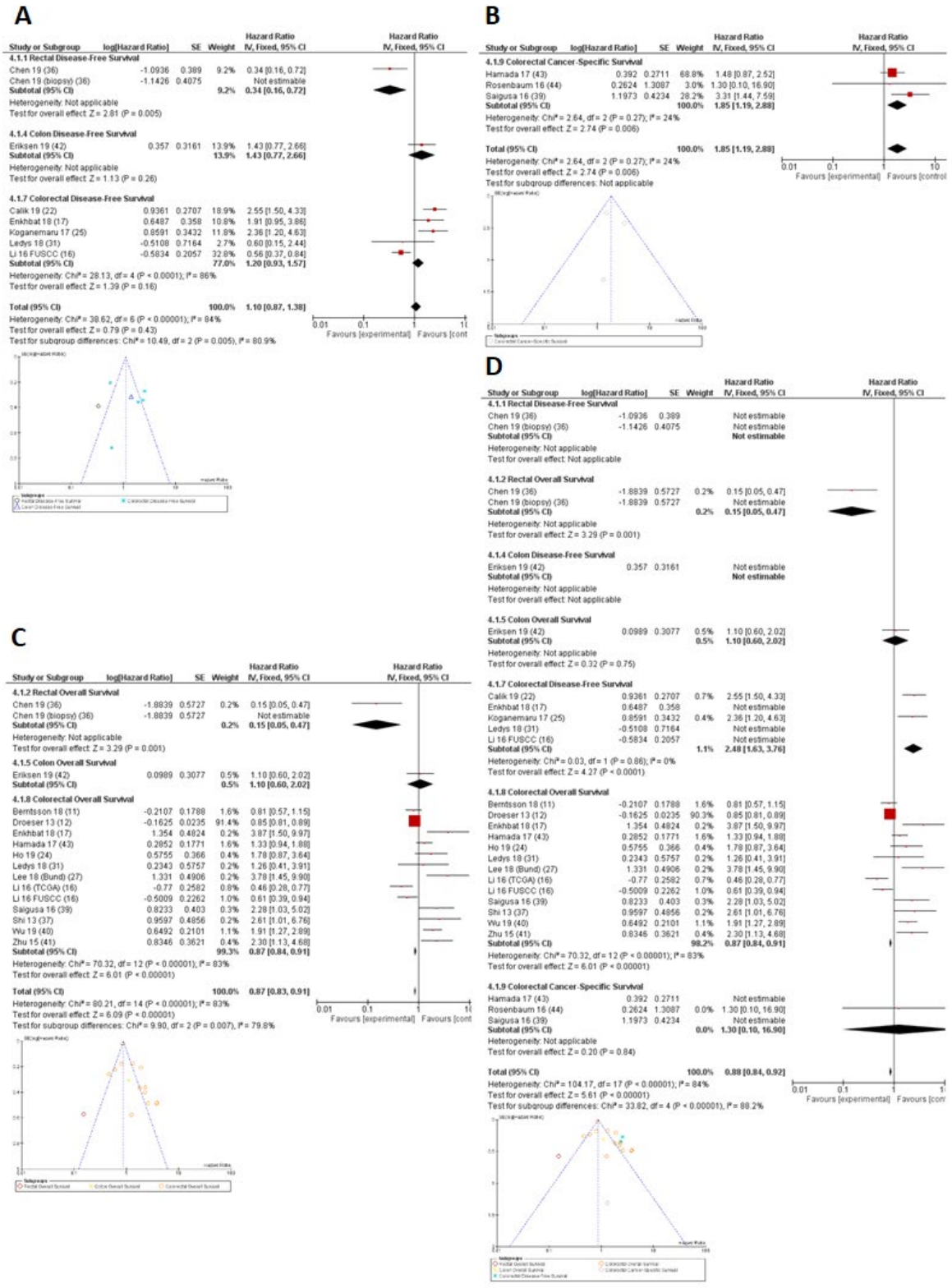


Figure 7.4. Forest plots and funnel plots for CD274 (PD-L1) expression on Tumour tissue for A) DFS, B) CSS and C) OS and D) All survival

7.3.4 CD274 (PD-L1) combined assessment in tumour tissue and immune cells

Four studies performed combined assessment of CD274 in tumour tissue and immune cells, comprising 835 patients(Bae et al., 2018, Hecht et al., 2016, Miller et al., 2017, Wei et al., 2018). Two studies (542 patients) found a significant beneficial survival impact(Hecht et al., 2016, Wei et al., 2018), whereas one (175 patients) found a significant detrimental survival impact(Bae et al., 2018). One (118 patients) found no impact on survival(Miller et al., 2017).

Two studies assessed for the presence of MSI, neither of which found MSI to be significant for survival(Miller et al., 2017, Wei et al., 2018).

All four studies met inclusion criteria for meta-analysis (Table 7.2, Figure 7.5) of which: two assessed DFS (HR 0.98; 95% CI: 0.71-1.34), with significant heterogeneity; one assessed CSS finding no significant survival association; two assessed OS (HR 1.06; 95% CI: 0.86-1.30), with significant heterogeneity; and all-cause survival (HR 1.00; 95% CI: 0.82-1.21) was significantly heterogeneous. Funnel plot analysis did not suggest any publication bias, although numbers were small (Figure 7.5).

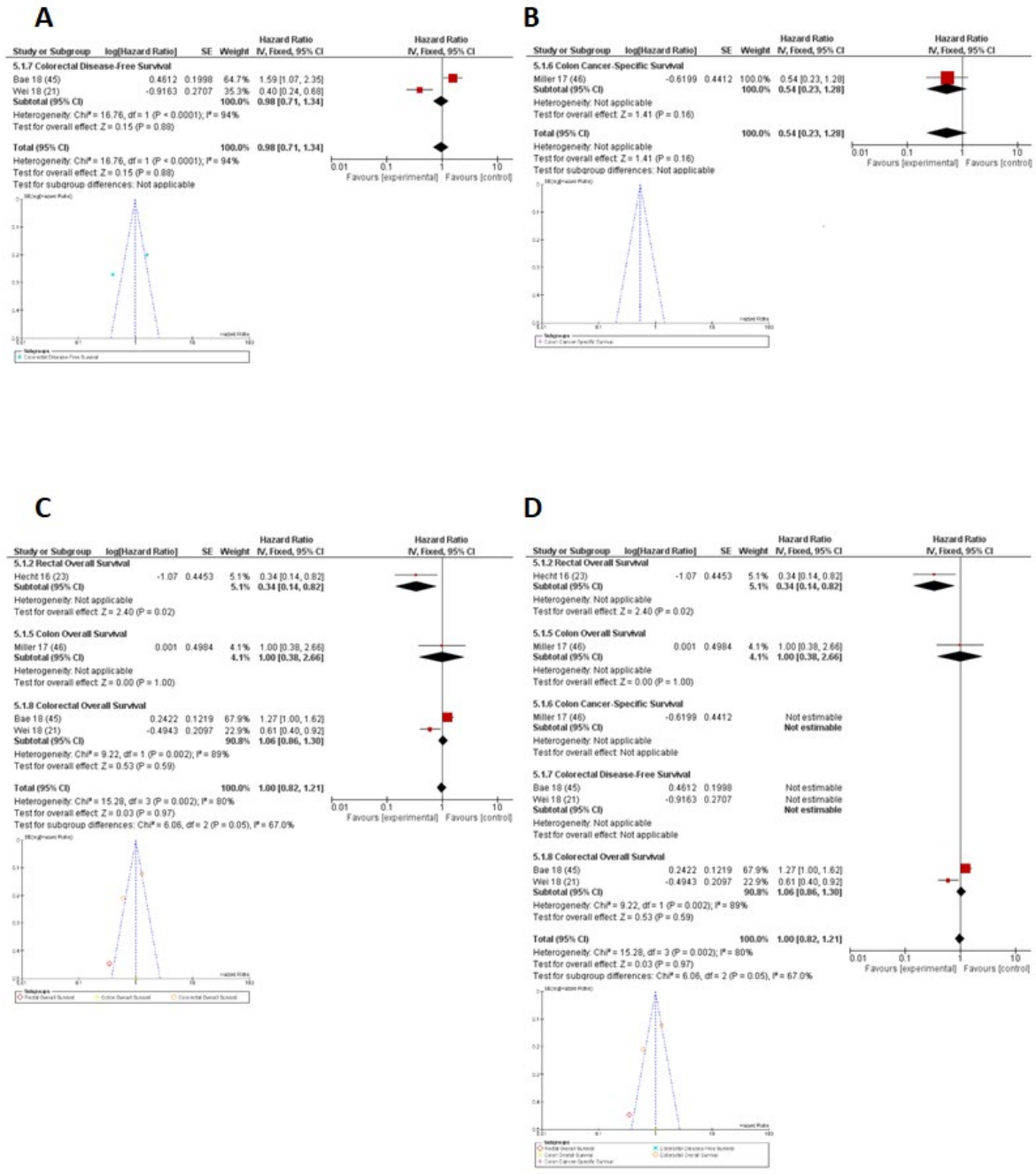


Figure 7.5. Forest plots and funnel plots for CD274 (PD-L1) expression on combined assessment of tumour tissue and immune cells for A) DFS, B) CSS and C) OS and D) All survival

7.3.5 CD274 (PD-L1) and response to anti-PD-1 therapy in CRC

There have been results published for 11 trials(Brahmer et al., 2012, Eng et al., 2019, Floudas et al., 2019, Hellmann et al., 2019, Le et al., 2015, Le et al., 2020, O'Neil et al., 2017, Overman et al., 2017, Yamamoto et al., 2017, Yarchoan et al., 2020, Chalabi et al., 2020) of anti-PD-1 therapy in CRC, as well as published abstracts with interim results for a further 20 trials(Andre et al., 2018, Azad et al., 2018, Boland et al., 2018, Callahan et al., 2017, Cassier et al., 2019, Chen et al., 2019a, Halama et al., 2019, Hochster et al., 2017, Hubbard et al., 2019, Lee et al., 2017b, Monjazeb et al., 2019, Patel et al., 2019, Rutkowski et al., 2019, Sanborn et al., 2018, Segal et al., 2016, Segal et al., 2019, Shahda et al., 2017, Shinozaki et al., 2018, Taylor et al., 2019) (Table 7.3). Of these, only 9 trials reported assessment of CD274 expression(Eng et al., 2019, Hellmann et al., 2019, Le et al., 2015, O'Neil et al., 2017, Overman et al., 2017, Yamamoto et al., 2017, Yarchoan et al., 2020, Sanborn et al., 2018, Chalabi et al., 2020). However, in 5 of these the number of individuals assessed for CD274 were either small(Yamamoto et al., 2017, Yarchoan et al., 2020, Sanborn et al., 2018), or the authors did not account for CD274 in survival analysis(Hellmann et al., 2019, Chalabi et al., 2020).

Le et al(Le et al., 2015) found CD274 to be expressed only on MSI tumours, which responded well to pembrolizumab. O'Neil et al(O'Neil et al., 2017) presented results for 23 patients, who all met inclusion criteria of tumour CD274 expression ≥1%, in a trial of Pembrolizumab in CRC. However, the only patient that responded to immunotherapy was an individual who also had MSI(O'Neil et al., 2017). Overman et al(Overman et al., 2017), in a cohort of 74 MSI CRC, took account both tumour CD274 status and immune cell CD274 status in response to Nivolumab monotherapy. They did not find any difference in response according to tumour CD274 expression but found that higher immune cell CD274 expression with a semiquantitative threshold was associated with better response(Overman et al., 2017). Eng et al(Eng et al., 2019), in the only phase III trial of anti-PD-1 therapy in CRC published

to date, randomised patients to receive Atezolizumab monotherapy, Atezolizumab + Cobimetinib or Regorafenib monotherapy. There were 363 patients overall of which 347 were confirmed MSS and 6 were confirmed MSI (3 in Atezolizumab arm and 3 in Atezolizumab + Cobimetinib arm). The Objective Response Rate (ORR) was 2% for both monotherapy arms and 3% for the combined arm. For MSI CRC in this trial, the response rate was 50%, with 1 of 3 responding in the Atezolizumab arm and 2 of 3 responding in the combined arm. Despite the low response rate in this trial, however, CD274 expression did appear to dictate response to therapy somewhat. In the Regorafenib monotherapy arm, low CD274 appeared to favour Regorafenib over either of the immunotherapy arms. Conversely, there was a non-significant trend towards high CD274 favouring both immunotherapy arms over Regorafenib(Eng et al., 2019).

Table 7.3. Anti-PD-1 therapy in colorectal cancer trials and the role of immunohistochemistry in predicting response

Trial (NCT)	Phase	CRC *N*	MSI *N*	MSS *N*	Treatment	CD274 assessed	Method	Threshold	Response
Published results									
Brahmer 2012 (NCT00729664) (Brahmer et al., 2012)	I	18	Unknown		Nivolumab monotherapy	N			ORR 0%
Chalabi 2020 (NCT03026140) (Chalabi et al., 2020)	II	60 planned	21	20	Neoadjuvant in early colon Ca: Nivolumab + Ipilimumab +- Cox-2	**Y**	IHC (Dako, 1:40) unclear assessment method	Uncl	MPR in 19 patients with MSI (60% pCR); MPR in 3 patients with MSS (13% pCR).
Eng 2019 (NCT02788279) IMblaze 370 (Eng et al., 2019)	III	363	6	347	Randomised controlled trial: Atezolizumab mono (3 MSI) vs Atezolizumab + Cobimetinib (3 MSI) vs Regorafenib mono	**Y**	IHC (Ventana, ?dilution) expressed on immune cells in either primary or metastasis	1%	ORR 2%, 3% and 2%, respectively. Survival was significantly better with Regorafenib in the CD274 low patients. High CD274 patients trended towards better survival in Atezolizumab or combined arms, but not significant. (ORR 50% for all MSI patients and atezolizumab +- Cobimetinib)
Floudas 2019 (NCT02298946) (Floudas et al., 2019)	I	15	Unknown	4 known	Amp-224 (anti-PD-1), cyclophosphamide + radiotherapy	N			ORR 0%
Hellmann 2019 (NCT01988896) (Hellmann et al., 2019)	Ib	84	2	62 (rest unknown)	Cobimetinib + atezolizumab	Y	IHC (Ventana, ?dilution) expressed on tumour tissue and immune cells in primary	5%	ORR 8% (50% for MSI patients and 10% in known MSS); no split per CD274 status given.

Le 2015 (NCT01876511) (Le et al., 2015)	II	28	10	18	Pembrolizumab monotherapy	Y	IHC (?antibody) membranous tumour cell expression	Uncl	IRORR 40% for MSI and 0% for MSS, CD274 only found to be expressed in MSI.
Le 2020 (NCT02460198) KEYNOTE 164 (Le et al., 2020)	II	124	124	0	Pembrolizumab monotherapy	N			ORR 33%
O'Neil 2017 (NCT02054806) (O'Neil et al., 2017)	Ib	23	1	22	Pembrolizumab monotherapy	Y	IHC (?antibody) membranous tumour expression or interface between immune cells/tumour	1%	ORR 4% (only patient with MSI responded), all had TPS of >1% for inclusion in trial
Overman 2017 (NCT02060188)* Checkmate-142 (Overman et al., 2017)	II	74 (314 planned)	74	0	Nivolumab monotherapy [vs Nivolumab + (Ipilimumab vs Cobimetinib vs anti-LAG3 vs Daratumumab) vs Nivolumab + Ipilimumab + Cobimetinib] **Results only for monotherapy arm**	Y	IHC (Dako, ?dilution), membranous tumour or immune cell staining (semiquantitative: rare, intermediate, numerous)	1%	ORR 31%; when split by CD274 expression; there were similar ORRs for tumour expression, whereas, in high immune cell expression, there was significantly better ORR (39% vs 24% vs 21% for numerous, intermediate and rare, respectively).
Yamamoto 2017 (NCT00441337) (Yamamoto et al., 2017)	I	4 (of 39 solid tumours)	Unknown (3 rectal, 1 colon)		Nivolumab monotherapy	Y	IHC (CD274, Medical&Biological laboratories Co, Nagoya, Japan) staining positive if same as positive control, no further detail.	SQ	ORR 25% (MSI unclear); 8 of 11 patients deemed CD274 high, unclear tumour type, all partial responders in this high group.
Yarchoan 2020 (NCT02981524) (Yarchoan et al., 2020)	II	17	0	17	GVAX, cyclophosphamide and pembrolizumab, single arm	Y	IHC (Spring Bioscience, ?dilution) tumour expression pre-treatment and subsequent biopsies (only 4 tested)	1%	ORR 0%, of 4 patients tested for CD274, all were initially low, although 1 became high on post-treatment biopsy, all four tumours displayed signs of necrosis on repeat biopsy

Poster results									
Andre 2018 (NCT02060188)* Checkmate-142 (Andre et al., 2018)	II	119	119	0	Nivolumab monotherapy [vs Nivolumab + (Ipilimumab vs Cobimetinib vs anti-LAG3 vs Daratumumab) vs Nivolumab + Ipilimumab + Cobimetinib] **Results only for Nivolumab + Ipilimumab arm**				ORR 55%
Azad 2018 (NCT02437136) ENCORE 601 (Azad et al., 2018)	II	16 (of 202 solid tumours)	0	16	Pembrolizumab + Entinostat (HDAC inhibitor)				IRORR 6%
Boland 2018 (NCT02713373) (Boland et al., 2018)	Ib/II	9	Unclear		Pembrolizumab + Cetuximab				ORR 0%
Callahan 2017 (NCT01975831) (Callahan et al., 2017)	I	11	Unclear		Durvalumab + Tremelimumab				ORR 9%
Cassier 2019 (NCT02777710) (Cassier et al., 2019)	I	14	Unclear (at least 2MSI)		Durvalumab + Pexidartinib (CSF-1R TKI)				ORR 0%
Chen 2019 (NCT02870920) (Chen et al., 2019a)	II	179	0	179	Standard chemo + Tremelimumab + Durvalumab vs Best supportive care (randomised)				Significantly better OS (median and disease control rate were 6.6months and 22.7% in experimental arm vs 4.1 and 6.6% in standard arm, respectively), although adverse events were higher in experimental arm.

Halama 2019 (NCT03168139) (Halama et al., 2019)	Ib/II	11	0	11	Pembrolizumab + Olaptesed pegol (CXCL12 inhibitor)				ORR 0%
Hochster 2017 (NCT01633970) (Hochster et al., 2017)	I	10 (of 240 solid tumours)	10	0	Atezolizumab + Bevacizumab arm presented (multiple other combinations)				ORR 30%
Hubbard 2019 (NCT03258398) (Hubbard et al., 2019)	II	56	0	56	Avelumab + Tomivosertib (MNK inhibitor)				ORR 2%
Lee 2017 (NCT02260440) (Lee et al., 2017b)	II	31	0	30	Pembrolizumab + Azacitidine				ORR 3%
Monjazeb 2019 (NCT02888743) (Monjazeb et al., 2019)	II	18	Unclear		Durvalumab + Tremelimumab + radiotherapy				ORR 0%
Patel 2019 (NCT02860546) (Patel et al., 2019)	II	18	0	18	Nivolumab + Trifluridine/Tipiracil				ORR 0%
Rutkowski 2019 (NCT02908906) (Rutkowski et al., 2019)	I/II	26	26	0	Cetrelimab monotherapy				ORR 8%
Sanborn 2018 (NCT02335918) (Sanborn et al., 2018)	I/II	42 (of 175 solid tumours)	Unclear		Nivolumab + Varlilumab (anti-CD27)	Y	Unclear		ORR 5% (2 of 41; 1 MSI, 1 MSS, both CD274 low)

Segal 2016 (NCT02437071) (Segal et al., 2016)	II	34	0	26	Pembrolizumab + radiotherapy OR radiofrequency ablation				ORR 9% (1 of 11) in radiotherapy arm, none in RFA arm
Segal 2019 (NCT01693562) (Segal et al., 2019)	I	36 (of 1022 solid tumours)	36	0	Durvalumab monotherapy				ORR 22%
Segal 2019 (NCT02227667) (Segal et al., 2019)	II	16	11	0	Durvalumab monotherapy				ORR 27% (unclear whether MSI or high tumour infiltrating lymphocytes)
Shahda 2017 (NCT02375672) (Shahda et al., 2017)	II	30	3	22	Pembrolizumab + mFOLFOX				ORR 64% (1 CR)
Shinozaki 2018 (NCT02851004) (Shinozaki et al., 2018)	Ib/II	94 MSS	0	12	Pembrolizumab + BBI608 (Napabucasin)				ORR 8% (1 of 12)
Taylor 2019 (NCT02811497) METADUR (Taylor et al., 2019)	II	14	0	14	Durvalumab + Azacitidine				ORR 0%

* same trial, results for different arms (sequential assignment)

Abbreviations: CRC, colorectal cancer; MSI, microsatellite unstable; MSS, microsatellite stable; ORR, objective response rate; MPR, major pathological response; pCR, pathological Complete Regression; IRORR, immune-related objective response rate; IHC, immunohistochemistry

7.4 DISCUSSION

It is clear that immune cell expression of PDCD1 or CD274 is, on the whole, associated with a beneficial impact on survival. However, when compared with various other immune cell assessments that have been validated for their prognostic role(Alexander et al., 2020b), it is not clear whether immune cell CD274 holds any additional value as a prognostic marker. When considering the expression of CD274 on tumour tissue the data are heterogenous with just as many studies/participants demonstrating a detrimental impact on survival as a beneficial impact and many studies finding no survival impact. Therefore, as a prognostic marker, tumour CD274 assessment appears to be of little value.

In terms of MSI, there is evidence of higher expression of CD274 in both tumour tissue and immune cells compared with MSS tumours(Lee et al., 2016, Le et al., 2015, Eriksen et al., 2019). In those studies assessing purely MSI patients, tumour CD274 expression was found to have either a detrimental survival impact(Lee et al., 2016) or no survival impact(Kim et al., 2016, Lee et al., 2017c, Lee et al., 2018b) and immune cell CD274 had either a beneficial survival impact(Lee et al., 2017c, Lee et al., 2018b) or no impact(Kim et al., 2016).

However, while CD274 may not be particularly useful as a prognostic marker, it may have a role in determining the efficacy of PD-1 checkpoint inhibitor therapy.

The first published trial assessing Nivolumab in solid tumours included 18 CRCs, but results were disappointing with an ORR of 0% in the CRC subgroup(Brahmer et al., 2012). Neither MSI status, nor CD274 expression were considered in this trial. However, there was a turning point for immunotherapy in CRC when Pembrolizumab was given to a variety of MSI cancers including 10 MSI CRC, as well as 18 MSS CRC, with a 40% Immune-Related ORR (IRORR) in the MSI arm, compared with 0% in the MSS arm(Le et al., 2015). This led to the American Food and Drug Administration (FDA) licensing Pembrolizumab for MSI CRC. Trials studying MSI CRC consistently report ORRs >30% for anti-PD-1

monotherapy(Overman et al., 2017, Le et al., 2020). These results in MSI are even more impressive when anti-PD-1 therapy is combined with other checkpoint inhibitors, with ORRs >50%(Andre et al., 2018).

Le et al(Le et al., 2015) also reported in the Pembrolizumab trial that CD274 expression was only present in the MSI patients studied, but postulated that MSS tumours expressing high levels of CD274 may also respond to anti-PD-1 therapy(Le et al., 2017). Following on from this O'Neil et al(O'Neil et al., 2017) presented results for a trial of largely MSS CRC in individuals with tumour CD274 expression of >1%. However, the only patient in this trial that responded to immunotherapy was an individual who also had MSI. Further trials of immunotherapy in MSS CRC have yielded poor results, alone or combined with other treatments.

The challenge, therefore, is to find a disease biomarker for MSS CRC that will identify those patients who will respond to anti-PD-1 therapy. The data on CD274 as a marker of disease response in CRC is sparse and the heterogeneity in CD274 assessment methodology among the published trials precludes any meaningful analysis.

In upper GI cancers, CD274 assessment as a biomarker of anti-PD-1 therapy response has been standardised, with a combined percentage score (CPS) threshold of >1 determining response to Pembrolizumab(Kulangara et al., 2019). However, a CPS of >10 has recently been described as a better biomarker for response to immunotherapy(Wainberg et al., 2020). The CPS method also uses a standardised antibody (22C3 pharmDx IHC assay)(Kulangara et al., 2019). One study in CRC compared the reproducibility of CD274 scoring using three different antibodies and multiple cut-points for survival(Lee et al., 2018a). There was wide variability in the quality of staining and therefore reproducibility of scoring depending on which antibody was used: those by Cell Signalling and Dako being the most specific of the three antibodies studied.

The other crucial question under investigation in multiple trials is whether the efficacy of immunotherapy in MSS CRC can be improved in combination with other treatments, such as radiotherapy or standard chemotherapy. Encouragingly, the interim results of a trial of standard chemotherapy vs standard chemotherapy with the addition of anti-PD-1 and CTLA4 inhibitors found response rates to be significantly better in the checkpoint inhibitor arm(Chen et al., 2019a). Furthermore, a single arm trial of Pembrolizumab + mFOLFOX with 3 MSI and 22 MSS CRC found an ORR of 64% (1 complete responder with MSI)(Shahda et al., 2017). Finally, a phase II trial of neo-adjuvant Nivolumab + Ipilimumab in early colon cancer found pathological complete response in 60% patients with MSI and 13% patients with MSS(Chalabi et al., 2020). There are a further 9 phase III trials assessing immunotherapy and CRC with no published results yet, in addition to many other phase I and II trials. A catalogue of all 223 registered trials to date comparing various different combinations of therapies with PD-1 inhibitors was made (Appendix 2.2).

Of course, it must be recognised that while the expression of PDCD1 and CD274 have been discussed largely in isolation in this review, there are many other factors that influence the expression of cell surface proteins at a genetic and epigenetic level. These factors include environmental stimuli, such as obesity, exercise, systemic inflammation, diet, smoking and the microbiome. The integration of cancer immunology and epidemiology research has been termed molecular pathology epidemiology (MPE) and has also been utilised alongside precision medicine to investigate the influence of environmental factors on treatment outcomes(Ogino et al., 2018, Hamada et al., 2019). Future studies should take account of environmental factors and their influence on cancer immunology and treatment response.

In conclusion, while CD274 expression on immune cells is largely associated with better survival, there are many other immune cell assessments that have been validated for their prognostic role and therefore immune cell CD274 adds little value as a prognostic marker. CD274 assessment in tumour tissue would appear to be of little use as a prognostic marker,

with significant inter-study heterogeneity and many studies finding no prognostic significance. As a marker for response to anti-PD-1 therapy in CRC, the data is sparse. CD274 expression analysis needs to be standardised moving forward. One strategy would be to adopt the CPS method already in use as a marker of response to immunotherapy in upper GI cancer. Once CD274 assessment is standardised, it may be possible to assess thresholds in clinical trials to determine if CD274 can select those MSS CRC patients who will respond to anti-PD-1 therapy. As in upper GI cancers, a CPS threshold of >10 is more likely to be selective for immunotherapy responders.

8. CONCLUSIONS

The TNM staging system is the cornerstone for basing clinical decisions. In terms of prognostic benefit, there is no staging or prognostic system that surpasses it. However, as has been reiterated many times before now, it stages only the tumour, and as medicine and cancer research is beginning to realise, the host's response to the tumour is just as important when selecting personalised treatments for each patient living with a cancer(Park et al., 2018).

To this end, there has been a wide-reaching search for additional prognostic biomarkers that will aid the personalisation cancer treatments. One such biomarker is the Glasgow Microenvironment Score.

In Chapter 1, an initial meta-analysis of the prognostic role of inflammatory cell assessments in colorectal cancer was performed(Alexander et al., 2020b). The results of this were that the presence of higher numbers or densities of almost all inflammatory cells, with the exception of macrophages (of certain subtypes) and cells expressing FoxP3, were significant for better survival outcomes. These findings require further assessment and work is already underway regarding macrophage subtypes by other groups(Shibutani et al., 2017a, De Palma and Lewis, 2013, Väyrynen et al., 2021). Furthermore, when compared alongside other inflammatory markers in colorectal cancer, strong KM grade performed well as a marker of good prognosis. It was also seen to compare with other immunohistochemical markers directly, both in the research contained herein (Chapter 4) and in research performed in other centres(Väyrynen et al., 2013). Therefore, a simple H&E-based score remains relevant as a marker of the immune phenotype.

However, it is not possible to use KM in all circumstances. For example, in pre-treatment staging biopsies obtained at colonoscopy, since it is not possible to score the tumour's invasive margin. Therefore, an alternative inflammatory cell stain, such as CD3 as a global T-cell marker, might be used in this setting, as described by Park et al(Park et al., 2020).

The advantages of KM over other inflammatory scores are its low cost and ready availability, given the fact that it can be performed on slides used routinely in histopathology to assess the TNM stage. It does not require any additional stains, nor the use of patented software or digital pathology, meaning that it is possible to score the GMS in an equitable manner in terms of global healthcare(Beaglehole and Bonita, 2010).

The GMS is by no means the only scoring system in CRC to incorporate both an inflammatory cell score and a mesenchymal score. Each of these scoring systems can be seen to have merit.

Hynes et al.(Hynes et al., 2017) published a prognostic score, the "fibroinflammatory score" in a dataset of 445 stage II-III colon cancers. They combined not only KM and TSP, but also added a further assessment of peritumoural inflammation, namely Crohn's-like reaction (CLR). Hynes et al.(Hynes et al., 2017) combined the "mild" and "none" CLR categories, comparing these with "intense" (see Chapter 1, section 1.5.3.2 for more background on CLR). The "fibroinflammatory" score assigned 1 point for weak KM, low CLR, or high stroma, giving a total possible score of 3 and a low score of 0. The fibroinflammatory score, like GMS, can be performed on standard H&E slides. The addition of a separate inflammatory score is not considered unreasonable given the literature concerning the positive impact of CLR on survival, but it is unclear why the two inflammatory scores were considered separately, while being given equal weighting. Furthermore, the score was only found to be significant for cancer-specific survival in patients with MSI high colon cancer and not in non-MSI tumours, which limits its relevance.

Li et al.(Li et al., 2018a) also proposed a combined inflammatory mesenchymal score. For inflammatory assessment, they used an IHC stain for macrophages (CD68) and for their mesenchymal assessment, they used the phenotypic feature of tumour budding. Tumour budding was associated with poor prognosis and strong local inflammatory response with good prognosis. Furthermore, they found that the presence of budding in the context of a

strong inflammatory infiltrate did not impact negatively on survival. This latter finding was similarly found when creating the GMS, since those with strong KM and high TSP did not have a statistically different cancer-specific survival than those with strong KM and low TSP(Park et al., 2015). However, since this score requires IHC staining to identify inflammatory cells, whereas the GMS uses only standard H&E-staining to achieve similar scores, the GMS was felt to have a slight advantage. In addition, the use of macrophages as markers of inflammatory infiltrate is not considered optimal, given the lack of consistency across multiple studies on meta-analysis(Alexander et al., 2020b) and the negative impact of the M2 phenotype.

One group has utilised an image-based neural network assessment in conjunction with deep-learning to generate the image-based Consensus Molecular Subtypes or (imCMS) comparing their machine learning with multi-omics data from three independent datasets(Sirinukunwattana et al., 2019). The imCMS had an impressive correspondence to omics characterisation of CMS with an AUC of 0.84 and 0.85 in the validation datasets. The advantage that GMS presently has over this technology is that it is readily available world-wide and requires no digital pathology or computer software. However, the use of digital pathology may indeed benefit the scoring of the GMS in the future, standardising this technique. Furthermore, studies comparing imCMS and GMS, in terms of correspondence with survival may be beneficial. As artificial intelligence is likely to become a larger part of histopathological scoring in the future(Cifci et al., 2022), this represents a further area of development for the GMS.

Genetic screening is extremely expensive and at the present time is not an economical means of guiding treatment(Cifci et al., 2022). Furthermore, there is insufficient knowledge regarding the broad range of mutations, apart from a select few that have well researched therapeutic targets, such as KRAS, BRAF and MSI and these are the main mutations discussed at MDT on a regular basis in current clinical practice in the United Kingdom.

Having said this, the benefits of genetic testing versus a phenotypic approach need to be assessed and an assessment of genetic mutations in each of the GMS phenotypes represents one of the future directions of the present research. Indeed recent research has suggested that a phenotypic assessment may guide which genetic mutations may be present in order to enable more targeted and economical mutational assessment(Cifci et al., 2022). Therefore, assessing genetic mutations associated with each GMS phenotype may further advance this field.

In Chapter 3, the relationship between GMS and EMT was assessed. GMS 0 was found to be associated with lower membrane Fascin expression, in addition to lower membrane and nuclear expression of B-catenin, whilst GMS 1 tumours were observed to have the highest membrane and cytoplasmic Fascin.

Since these are novel findings and no other studies to date have assessed peritumoural inflammation in the context of EMT, the nature of these findings is unclear, although it is hoped that with mutational data, it may be possible to explain these findings. It may be that, whilst not directly linked, the strong peritumoural inflammation is protective in GMS 0 tumours in spite of loss of membrane B-catenin.

In GMS 2, there were greater numbers of tumours expressing high nuclear B-catenin, in keeping with a mesenchymal phenotype. However, on the whole, there were fewer associations between EMT markers and GMS 2 than originally hypothesised. This hypothesis was based on the premise that higher TSP reflects EMT in the tumour. However, high TSP may in fact reflect the ability of the tumour to recruit the hosts local fibroblasts and transform these by a variety of chemokines to enable the negative pathological features associated with high TSP, such as neoangiogenesis(Conti and Thomas, 2011, McCorry et al., 2018).

These associations between EMT and different GMS categories have not previously been demonstrated and therefore warrant further investigation in independent cohorts. Genetic assessment of each GMS category may shed further light on these associations with markers of EMT.

The GMS was subsequently validated in a number of independent cohorts in Chapter 4. These cohorts found, universally, that GMS 0 had a more favourable prognosis compared with GMS 1 or 2. Analysis was performed in a variety of ways. For example, given the anecdotal difficulties with measuring TSP in rectal cancers post neoadjuvant chemoradiotherapy (which may induce a fibrotic reaction in the tissues and may make distinction of TSP more difficult(Hav et al., 2015)), colon cancers were selected out for assessment in the AP TMA cohort. In this analysis, however, those with GMS 1 had a particularly poor outcome. It was unclear whether this was due to the exclusion of the rectal cancers or whether it reflected other differences between the original GRI patients and the patients in this retrospectively collated dataset. Therefore, the same analysis in colon cancers alone was performed in a further independent group of prospectively collected GRI patients (the GRI-CRC-TMA cohort). This analysis showed that GMS was able to stratify survival into three distinct bands as in the original study. Further interrogation of the AP TMA cohort revealed a significantly higher proportion of emergency presentations, particularly in the GMS 1 group, which may account for the reversal of GMS 1 and GMS 2 in certain subgroup analyses for the AP TMA.

Validation in colon and rectal cancers in the combined JP-AP TMA cohort, whilst not a true validation given the inclusion of some of the cases from the original study, revealed the same stratification as the original study. However, of more value in this cohort was the ability to assess survival analysis in subcategories, given the increase in overall tumour numbers. This subgroup analysis showed that GMS was able to stratify CSS in low-risk and high-risk TNM

stage, and also in both rectal and colon cancers. In overall survival, GMS was able to stratify the whole cohort, but was only able to stratify high-risk TNM and colon cancer subgroups.

The final validation cohort (TransScot) assessed survival using Disease-free Survival, a measure akin to overall survival, but also including any evidence of disease recurrence. GMS was able to stratify survival in this cohort of TNM III cancers as a whole. Similar to the findings with overall survival in the combined JP-AP TMA, GMS also clearly stratified both higher-risk TNM III and colon cancers. However, there was still a significant difference GMS 0 vs GMS 2 in lower-risk TNM.

In Chapter 5, recurrence risk and pattern was assessed according to GMS. GMS was able to stratify risk of recurrence at any location in colorectal cancer as a whole and this was independent on multivariate analysis for rectal cancers, but not for colon cancers. The numbers of local recurrences were smaller, particularly in the rectal cancer subgroup and GMS did not reach univariate significance in this subgroup. However, in the colon cancer subgroup, GMS was significant on univariate analysis, although not independent of T-Stage or N-stage on multivariate analysis.

GMS 0 cancers had the lowest recurrence rate, although the recurrence rate was not zero and whilst strong peritumoural inflammatory response is considered protective, there are clearly other factors at play in this group. Of note, the type of immune cells is not accounted for by this specific scoring system. Others have shown that polarisation of macrophages to M2 macrophages may be a poor prognostic sign(Väyrynen et al., 2021). It has been previously shown that colorectal cancer recurrences in the immune subgroup may have a worse prognosis than in other subgroups(Testa et al., 2020). These, therefore, represent areas requiring further investigation and the combination of genetic profile and GMS is one of the planned future directions of study.

GMS 2 cancers had the highest rates both of local and distant disease recurrence. Previous work suggests that this mesenchymal phenotype, characterised by high TSP and accompanied by a poor immune response, denotes a mesenchymal subtype with poor prognosis and higher recurrence risk. There are several confounding factors in this group, since high TSP tumours are known to be associated with more advanced T-stage, N-stage and venous invasion, a finding also demonstrated in other studies. Whilst not independent of T-stage and N-stage for local disease recurrence, GMS was an independent prognostic marker when comparing overall risk of recurrence at any location. The lack of independence of GMS for local recurrence may be partly due to the lower numbers of local recurrences.

Given the high-risk nature of the GMS 2 phenotype, these tumours warrant more aggressive follow up with an enhanced surveillance programme, in order to detect recurrent disease at an earlier stage.

In Chapter 6, GMS and associations with chemotherapy were assessed. This was firstly performed on the prospectively collected data for the GRI-CRC-TMA. Analysis was limited in this cohort since the type and duration of chemotherapy were unknown. In spite of the limitations identified, there were some interesting findings in this cohort. Patients receiving conventional chemotherapy with a GMS 2 phenotype did not have a significantly better survival than those who did not receive any chemotherapy, in spite of evidence of greater comorbidities in the latter subgroup.

A more comprehensive assessment of the associations between GMS and standard chemotherapy type and duration was subsequently performed using the TransScot data. The only category in which GMS was able to distinguish between CAPOX and FOLFOX was GMS 0. This immune high phenotype appeared to benefit from FOLFOX over CAPOX. Similarly, patients in the French arm of the IDEA trial with higher immune infiltrates were

identified as benefiting from longer duration of FOLFOX(Pagès et al., 2020), although in the current research, duration did not seem to vary with GMS category.

The research contained herein, whilst not conclusive, lends further weight to the evidence that GMS 2 tumours do not respond well to conventional adjuvant chemotherapy regimens. Further research is ongoing regarding different subtypes of cancer associated fibroblasts and the challenges to chemoresistance these pose(Menezes et al., 2022). In addition, further clinical trials are ongoing regarding high stromal tumours and alternative therapies that may target these tumours.

Certain mutations in colorectal cancer are known to convey better response to neo-adjuvant and adjuvant treatments and can offer targeted therapies, such as immunotherapies in recurrent MSI high colorectal cancer. Regorafenib (a multikinase inhibitor) and Bevacizumab (a VEGF antagonist) have both shown some promise in high stromal tumours, believed to play a role in inhibiting neoangiogenesis (Grothey et al., 2019, Zunder et al., 2019, Fridman et al., 2020).

However, the optimal means to select which colorectal cancers might respond better to specific measures is far from being conclusively established. Clinical trials in this field are ongoing(McGregor and Price, 2019).

In Chapter 7, the evidence surrounding PDL1 (CD274) assessment in CRC was explored in a final meta-analysis. The evidence for PDL1 as a marker of response to immunotherapy in colorectal cancers is relatively poor at present. As a marker of prognosis, it depends in which tumour microenvironmental locus the molecule is measured. Whilst the presence of the protein on the surface of lymphocytes is associated with a good prognosis, this is likely to be a simple factor of the presence of increased density of immune cells. However, the

evidence for PDL1 as measured in tumour cells was equivocal in terms of prognostic relevance.

Further research is required in this area regarding PDL1 as a marker of disease response to immunotherapies in colorectal cancer. In addition, PDL1 assessment with regard to multiplex immunohistochemical assessment of the CRC tumour microenvironment may give additional information with regard to the spatial association of inflammatory cells to tumour cells and understanding this molecule and its role in immune escape(Lazarus et al., 2018).

In summary, the present thesis suggests that the Glasgow Microenvironment Score, based on pathological assessment of both immune and mesenchymal phenotypes, represents both a validated prognostic tool and also an effective means of detecting tumours with a propensity to recur. In addition, the use of this simple score may, with further clinical trial data, be able to identify specific classes of chemotherapy in order to target the specific tumour phenotypes assessed.

GMS 2 itself represents a poor prognostic feature, even in early-stage disease, with a higher risk of both local and systemic recurrence and therefore should be considered an additional high-risk feature warranting consideration for further adjuvant chemotherapy. GMS 2 tumours also warrant more aggressive follow up strategies to detect recurrences early.

Some interesting novel associations between EMT markers and the GMS were identified that warrant further exploration in independent cohorts and comparison with mutational data.

Further work exploring the genetic mechanisms underlying the different GMS phenotypes to consolidate these and, in addition, to validate their associations with the consensus molecular subtypes would further enhance the relevance of the GMS moving forwards. The

role of artificial intelligence may further consolidate the GMS and mutational differences between GMS subtypes represents an ongoing area of research.

APPENDICES

Appendix 1. Tables representing data mined from papers in systematic review and meta-analysis of inflammatory cells and prognosis in colorectal cancer (Chapter 1).

Table A1.1. Characteristics of studies assessing KM grade/Jass in rectal, colon and colorectal cancer

Rectal cancer														
Study	**TMA or Whole section**	**If TMA …**			**Anti-bodies used**	**Cohort assessed (stage)**	**Measu-rement**	**Threshold**	**Tumour region**	**Groups (overall or high to low)**	**Significant**	**UV or MV**	**MMR**	**Median follow up**
		How many cores?	**Size**	**Choice of core**										
Disease-free survival														
Szynglarewicz et al 2007	Whole					II – III	Jass	Semiquantitative	IM	13 vs 32	Y	UV [a]	NA	Unclear, 5-years quoted
Colon cancer														
Overall survival														
Hynes et al 2017	Whole					II – III	KM	1-2 vs 3-4	IM	392 vs 53	Y	MV	99 of 408	5.5 years
Disease-specific survival														
Hynes et al 2017	Whole					II – III	KM	1-2 vs 3-4	IM	392 vs 53	Y	MV	99 of 408	5.5 years
Colorectal cancer														
Disease-free survival														
Coca et al 1997	Whole					I – III	Jass	Semiquantitative	IM	62 vs 84 vs 11	Y	MV	NA	Unclear, 5-

														years quoted
Nagtegaal et al 2001	Whole					II – IV (160: 40 Stage II, 40 stage III, 80 stage IV)	Jass	Semiquantitative	IE+ST	1415 overall (2 groups)	Y	UV [b]	NA	19.6 months
Menon et al 2004	Whole					II – III	Jass	Semiquantitative	IE+ST+ IM	15 vs 43 vs 35	NS		6 of 90	6.1 years
Klintrup et al 2005	Whole					I – II	Semiquantitative	0-2 vs 3-4	IE+ST	62 vs 167	NS		11 of 99	41 months
									IM	128 vs 100	Unclear [c]			
Huh et al 2012	Whole					I – IV	KM	0-2 vs 3-4	IM	442 vs 104	NS		NA	54 months
Jakubowska et al 2017	Whole					I – IV	KM	0-2 vs 3-4	IM	73 vs 86	Y	UV [d]	NA	24-30 months
Shibutani et al 2018	Whole					IV	Manual	>50% "TILS"	IM	30 vs 27	NS		NA	Unclear
Climent et al 2019	Whole					I – III (MSI High)	KM	0-2 vs 3-4	IM	146 vs 27	Y	UV	173 of 173	Unclear, 60-months quoted
Overall survival														
Coca et al 1997	Whole					I – III	Jass	Semiquantitative	IM	62 vs 84 vs 11	Y	MV	NA	Unclear, 5-years quoted
Nagtegaal et al 2001	Whole					II – IV (160: 40 Stage II, 40 stage III, 80 stage IV)	Jass	Semiquantitative	IE+ST	1415 overall (2 groups)	Y	UV [e]	NA	19.6 months

Cianchi et al 2002	Whole					I – II	Jass	Semiquantitative	IM	16 vs 68	NS		NA	67
Klintrup et al 2005	Whole					I – II	Semiquantitative	0-1 vs 3-4	IE+ST	62 vs 167	NS		11 of 99	41 months
									IM	128 vs 100	Y	MV		
Gao et al 2005	Whole					I – IV	Jass	Semiquantitative	IE+ST	39 vs 262	Unclear		25 of 177	Unclear
									IM	64 vs 237	Y	MV		
Ogino et al 2009	Whole					I – IV	Jass	Semiquantitative	IM	93 vs 707 vs 43	Y	MV	124 of 826	Unclear, 10-years display
Kasajima et al 2010	Whole					Stage I-IV CRC	Unclear	Semiquantitative	Unclear	103 vs 181	NS		NA	38.4 months
Bae et al 2011 (2 cohorts)	Whole					I – IV (MSIH)	Jass	Semiquantitative	IM	145 vs 24	NS		All 169	38/ 53 months
Huh et al 2012	Whole					I – IV	KM	0-2 vs 3-4	IM	442 vs 104	Y	MV	NA	54 months
Shibutani et al 2018	Whole					IV	Manual	>50% TILS	IM	30 vs 27	Y	MV	NA	Unclear
Xie et al 2018	Whole					IV	Jass	Semiquantitative	IE+ST+ IM	197 vs 105	Y	MV	14 of 302	27.7 months
Climent et al 2019	Whole					I – III (MSI High)	KM	1-2 vs 3-4	IM	146 vs 27	Y	UV	173 of 173	Unclear, 60-months quoted
Disease-specific survival														
Chiba et al 2004	Whole					I – IV	Manual	Semiquantitative (split: prominent vs mod/low)	IM	60 vs 311	Y	MV	36 of 366	7.7years

Ogino et al 2009	Whole					I – IV	Manual	Semiquantitative	IM	62 vs 168 vs 613	Y	UV [f]	124 of 826	Unclear, 10-years display
Park et al 2014	Whole					I – IV	KM	0-1 vs 3-4	IM	103 vs 204	Y	MV	NA	Unclear, 5-years quoted
Väyrynen et al 2014	Whole					I – IV	KM	Semiquantitative	IM	329 overall (2 groups)	Y	MV	36 of 398	Unclear, 60-months quoted

[a]nil significant on MV [b]not independent of mast cells/eosinophils [c]no data given for RFS [d]for MV, CI crosses 1.0 [e]not independent of mast cells/eosinophils [f]not independent of KRAS/BRAF/p53/LINE-1/MSI/CIMP

Table A1.2. Characteristics of studies assessing CLR/plasma cells in colon and colorectal cancer

Study	TMA or Whole section	If TMA …			Anti-bodies used	Cohort assessed (stage)	Measu-rement	Threshold	Tumour region	Groups (overall or high to low)	Significant	UV or MV	MMR	Median follow up
		How many cores?	Size	Choice of core										
Colon cancer														
Overall survival														
Hynes et al 2017	Whole					II – III	Graham-Appelman	0 vs 1-2	IM	292 vs 153	Y	MV	99 of 408	5.5 years
Disease-specific survival														
Harrison et al 1995	Whole					I – III (Right sided)	Graham-Appelman	0-1 vs 2	IM	96 vs 248	Y	MV	NA	Unclear, 5-years quoted
Hynes et al 2017	Whole					II – III	Graham-Appelman	0 vs 1-2	IM	292 vs 153	Y	MV	99 of 408	5.5 years
Colorectal cancer														
Disease-free survival														
Buckowitz et al 2005	Whole					I – IV	Graham-Appelman	0-1 vs 2	IM	42 vs 76	NS		47 of 120	33 months
Klintrup et al 2005	Whole					I – IV	Graham-Appelman	0 vs 1-2	IM	40 vs 138	NS		11 of 99	41 months
Kim et al 2015	Whole					I – IV (MSI High)	Graham-Appelman	0-1 vs 2	IM	48 vs 164	NS		212 of 212	4.96 years
							Ueno criteria	>1mm size		86 vs 126	Y	MV		
							Väyrynen criteria	>3.8/mm density		138 vs 74	Y	MV		
Ueno et al 2015	Whole					I – IV	Ueno criteria	>1mm size	IM	695 vs 185	Y	MV	NA	68 months
										297 vs 177	Y	MV		

Lee et al 2016	Whole					I – IV	No. of lymphoid aggregates with >5 as severe	Semiquantitative	IE+ST	1 vs 160 vs 230	Y	UV[a]	68 of 389	55 months
Overall survival														
Nielsen et al 1999	Whole					I – IV	Electronic (GRID, Olympus)	Quartiles	IE+ST	174 vs 143 vs 126 vs 145	Y	UV		61 months
Buckowitz et al 2005	Whole					I – IV	Graham-Appelman	0-1 vs 2	IM	42 vs 76	Y	UV	47 of 120	33 months
Klintrup et al 2005	Whole					I – IV	Graham-Appelman	0 vs 1-2	IM	40 vs 138	NS		11 of 99	41 months
Ogino et al 2009	Whole					I – IV	Semiquantitative	Semiquantitative	IM	62 vs 168 vs 613	Y	MV	124 of 826	Unclear, 10-years display
Bae et al 2011 (2 cohorts)	Whole					I – IV (MSIH)	Graham-Appelman	0 vs 1-2	IM	144 vs 25	Y	MV	All 169	38 or 53 months
Rozek et al 2016	Whole					I – IV	Graham-Appelman	0-1 vs 2	IM	784 vs 879	Y	MV	318 of 2149	Unclear, 10-years display
Disease-specific survival														
Ogino et al 2009	Whole					I – IV	Graham-Appelman	0 vs 1 vs 2	IM	62 vs 168 vs 613	Y	UV[d]	124 of 826	Unclear, 10-years display
Richards et al 2012	Whole					I – III	Manual	Median	IM	65 vs 65	Y	UV[e]	NA	105 months
Ueno et al 2013 (training)	Whole					I – IV	Ueno criteria	>1mm size	IM	210 vs 822	Y	MV	17 of 225	Unclear, 5-years quoted
Ueno et al 2013 (validation)	Whole					I – IV	Ueno criteria	>1mm size	IM	186 vs 314	Y	MV	NA	68 months

Väyrynen et al 2014	Whole					I – IV	Väyrynen criteria	Roc curve (>0.38/mm density)	IM	329 overall (2 groups)	Y	MV	36 of 398	Unclear, 60-months quoted
Rozek et al 2016	Whole					I – IV	Graham-Appelman	0-1 vs 2	IM	784 vs 879	Y	MV	318 of 2149	Unclear, 10-years display

[a]no MV for whole cohort shown [b]not independent of N-stage/M-stage/age (also numbers small in positive group) [c]no MV given

[d]not independent of KRAS/BRAF/p53/LINE-1/MSI/CIMP [e]not independent of KM grade

Table A1.3. Characteristics of studies assessing TILs on H&E in colon and colorectal cancer

Study	TMA or Whole section	If TMA …			Anti-bodies used	Cohort assessed (stage)	Measu-rement	Threshold	Tumour region	Groups (overall or high to low)	Significant	UV or MV	MMR	Median follow up
		How many cores?	Size	Choice of core										
Colon cancer														
Disease-free survival														
Turner et al 2016	Whole					II	Manual	Semiquantitative	IE	167 vs 229	Y	UV[a]	25 of 196	5.1 years
Overall survival														
Turner et al 2016	Whole					II	Manual	Semiquantitative	IE	167 vs 229	Y	MV	25 of 196	5.1 years
Colorectal cancer														
Disease-free survival														
Ropponen et al 1997	Whole					I – IV	Manual	Semiquantitative	ST+IM	26 vs 54 vs 106 vs 9	Y	MV	NA	14 years
Lee et al 2016	Whole					I – IV	Manual	Semiquantitative	IE+ST	35 vs 109 vs 175 vs 74	Y	UV[b]	68 of 389	55 months
Jakubowska et al 2017	Whole					I – IV	Manual	<20%>	ST	75 vs 85	NS		NA	24-30 months
								Present/ absent	IE	124 vs 36	NS			
Iseki et al 2018	Whole					II – III	Electronic (Micro-analyzer, JPDC)	Roc curve	ST	42 vs 118	Y	MV	9 of 156	63.5
Climent et al 2019	Whole					I – III (MSI High)	Unclear	Unclear	IE+ST	128 vs 37	NS		173 of 173	Unclear, 60

														months quoted
Overall survival														
Nielsen et al 1999	Whole					I – IV	Electronic (GRID, Olympus)	Quartiles	IE+ST	163 vs 160 vs 135 vs 130	Y	UV		61 months
Klintrup et al 2005	Whole					I – II	Manual	Semiquantitative	IE+ST	76 vs 152	Y	UV[c]	11 of 99	41 months
									IM	128 vs 99	Y	UV[c]		
Ogino et al 2009	Whole					I – IV	Manual	Semiquantitative	IE	96 vs 123 vs 624	NS		124 of 826	Unclear, 10-years display
									ST	97 vs 709 vs 37	Y	MV		
Rozek et al 2016	Whole					I – IV	Manual	>2TILs/ HPF	IE	621 vs 1647	Y	MV	318 of 2149	Unclear,10-years display
Iseki et al 2018	Whole					II – III	Electronic (Micro-analyzer, JPDC)	Roc curve	ST	42 vs 118	Y	MV	9 of 156	63.5
Lang-schwartz et al 2018	Whole					I – IV	Manual	Median	IE+ST	230 vs 271	Y	UV[c]	NA	42 months
Climent et al 2019	Whole					I – III (MSI High)	Unclear	Unclear	IE+ST	128 vs 37	NS		173 of 173	Unclear, 60-months quoted
Disease-specific survival														
Ropponen et al 1997	Whole					I – IV	Manual	Semiquantitative	ST+IM	26 vs 54 vs 106 vs 9	Y	MV	NA	14 years
Ogino et al 2009	Whole					I – IV	Manual	Semiquantitative	IE	96 vs 123 vs 624	NS			

									ST	97 vs 709 vs 37	Y	MV	124 of 826	Unclear, 10-years display
Richards et al 2012	Whole					I – III	Manual	Median	IE	65 vs 65	Y	UV[d]	NA	105 months
Rozek et al 2016	Whole					I – IV	Manual	>2TILs/ HPF	IE	621 vs 1647	Y	MV	318 of 2149	Unclear,10-years display

[a]not independent of NLR/T4/LVI [b]not independent of TNM/PD1 expression [c]no MV given [d]not independent of KM grade

Table A1.4. Characteristics of studies assessing Combined H&E inflammatory infiltrate in colorectal cancer

Study	TMA or Whole section	If TMA …			Cohort assessed (stage)	Measurement	Threshold	Tumour region	Groups (overall or high to low)	Significant	UV or MV	MMR	Median follow up
		How many cores?	Size of cores	Choice of core									
Overall survival													
Ogino et al 2009	Whole				I – IV	CLR/KM/stromal and intraepithelial TILs	Arbitrary	IE+ST+IM	64 vs 230 vs 549	Y	MV	124 of 826	Unclear, 10-years display
Wallace et al 2018 (Caucasian American cohort)	Whole				I – IV	As per Ogino et al 2009	Arbitrary	IE+ST+IM	159 overall (4 groups)	NS		22 of 139	Unclear, no indication
Wallace et al 2018 (Afro-American cohort)	Whole				I – IV	As per Ogino et al 2009	Arbitrary	IE+ST+IM	52 overall (4 groups)	Y	MV[a]	3 of 50	Unclear, no indication
Disease-specific survival													
Ogino et al 2009	Whole				I – IV	CLR/KM/stromal and intraepithelial TILs	Arbitrary	IE+ST+IM	64 vs 230 vs 549	Y	MV	124 of 826	Unclear, 10-years display

[a]independent of MSI

Table A1.5. Characteristics of studies assessing CD3 in rectal, colon and colorectal cancer

Study	TMA or Whole section	If TMA …			Anti-bodies used	Cohort assessed (stage)	Measu-rement	Threshold	Tumour region	Groups (overall or high to low)	Significant	UV or MV	MMR	Median follow up
		How many cores?	Size	Choice of core										
Rectal cancer														
Disease-free survival														
Teng et al 2015	Biopsy				ZM-0508, Beijing ZGBBC	II – III	Manual	Mean	ST	75 vs 61	Y	UV[a]	NA	57 months
McCoy et al 2017	Biopsy				Dako	II – IV	Electronic (Strata-quest V5)	Median	IE+ST	46 vs 47	NS		NA	78 months
Overall survival														
Teng et al 2015	Biopsy				ZM-0508, Beijing ZGBBC	II – III	Manual	Mean	ST	75 vs 61	Y	UV[a]	NA	57 months
Disease-specific survival														
McCoy et al 2017	Biopsy				Dako	II – IV	Electronic (Strata-quest V5)	Median	IE+ST	46 vs 47	NS		NA	78 months
Colon cancer														
Disease-free survival														
Guidoboni et al 2001	Whole				Dako	II – III (Right-sided)	Electronic	Median	IE	109 overall (2 groups)	Y	MV[b]	47 of 109	78 months
	TMA	3				II – III	Manual		IE	113 vs 47	Y	MV		

Sinicrope et al 2009			Unclear	Representative	F7.2.38, Dako			1st quartile	ST	120 vs 40	NS		13 of 125	Unclear, 5-years quoted
Lee et al 2010	Whole				Thermo Fisher Scientific	II	Electronic	Mean	IE+ST	23 vs 38	Y	UV[c]	NA	125 months
Flaherty et al 2016	Whole				2GV6, Ventana MST	I – III	Electronic	Contin-uous	IM	89 overall (2 groups)	Y	UV[a]	NA	33.6 months
								25th %ile	IE+ST+IM	89 overall (2 groups)	Y	UV[d]		
Overall Survival														
Guidoboni et al 2001	Whole				Dako	II – III (Right-sided)	Electronic	Median	IE	109 overall (2 groups)	Y	MV[b]	47 of 109	78 months
Sinicrope et al 2009	TMA	3	Unclear	Repres-entative	F7.2.38, Dako	II – III	Manual	1st quartile	IE	113 vs 47	NS		13 of 125	Unclear, 5-years quoted
									ST	120 vs 40	NS			
Lee et al 2010	Whole				Thermo Fisher Scientific	II	Electronic	Mean	IE+ST	23 vs 38	Y	UV[c]	NA	125 months
Peng et al 2010	Whole				Zymed	III b	Manual	Arbitrary, > 75% staining	ST	51 vs 17	Y	UV[e]	NA	Unclear, 5-years quoted
Miller et al 2017	TMA	3	1mm	2xCT, 1xIM	2GV6	III	Electronic (Aperio)	Data driven	IE+ST	87 vs 17	NS		18 of 104	82.5 months
									IM	44 vs 12	NS			
Disease-specific survival														
Miller et al 2017	TMA	3	1mm	2xCT, 1xIM	2GV6	III	Electronic (Aperio)	Data driven	IE+ST	87 vs 17	Y	MV	18 of 104	82.5 months
									IM	44 vs 12	NS			
Colorectal cancer														

Disease-free survival													
Nagtegaal et al 2001	Whole				Dako	II – IV (160: 40 Stage II, 40 stage III, 80 stage IV)	Manual	Unclear	IE+ST	32 vs 42 vs 76	Y	MV	NA	35.4 months
									IM	47 vs 41 vs 63	Y	UV[f]		
Galon et al 2006	TMA	4	0.6mm	2x CT,	SP7, Neomarkers	I – III	Electronic (Alphelys)	ROC curve	IE+ST+IM	124 vs 95 vs 30	Y	MV	NA	45.3 months
			1mm	2x IM										
Laghi et al 2009	Whole				F7.2.38, M7254; Dako	II – III	Electronic	ROC curve (>10% vs <1%)	IM	41 vs 77	Y	MV	48 of 286	74 months
						(Node-negative)		Median		74 vs 74	Y	UV[a]		
Deschoolmeester et al 2010	Whole				SPF7, Neomarkers	I – IV	Manual	Semiquantitative	IE	64 vs 141	Y	UV[g]	27 of 215	4.9 years
									ST	82 vs 133	NS			
									IM	150 vs 65	NS			
Katz et al 2013	TMA	3	0.6mm	Liver Met	F7.2.38, Dako	IV	Electronic (Aperio)	Optimal *p*-value	IE+ST	38 vs 149	NS		NA	95 months
Lavotshkin et al 2015	Whole				2GV6, Ventana MST	I – III	Electronic	Unclear	IE+ST+IM	35 overall (2 groups)	NS		NA	46.8 months
Chen et al 2016	TMA	2	1mm	Representative	ZSGB-BIO	I – IV	Electronic (TMAJ, JHU)	ROC curve	IE+ST	300 overall (2 groups)	Y	UV[a]	NA	62.9 months
Väyrynen et al 2016	TMA	1-4	3mm	IM + CT	PS1, Novocastra	I – IV	Electronic (Image J)	ROC curve	IE	147 overall (2 groups)	Y	MV	11 of 147	51 months
									ST	147 overall	NS			

										(2 groups)				
									IM	147 overall (2 groups)	NS			
Schweiger et al 2016	Pulmo-nary Met				SP7, #RM 9107-S1, Thermo Fisher Scientific	IV	Manual	Semiquantitative	IE+ST	27 vs 28	NS		NA	30 months
									IM	32 vs 23	NS			
Kim et al 2018	TMA	1	2mm	IM	Dako	I – IV	Electronic (Aperio)	Median	IE	329 vs 327	Y	UV[h]	44 of 488	Unclear, 80-months display
									ST	320 vs 334	Y	UV[h]		
Eriksen et al 2018	Whole				M7254, Dako	II	Electronic	Lower tertile	IE+ST	387 vs 186	Y	MV	173 of 573	7 years
Overall Survival														
Nagtegaal et al 2001	Whole				Dako	II – IV (160: 40 Stage II, 40 stage III, 80 stage IV)	Manual	Unclear	IE+ST	32 vs 42 vs 76	Y	UV[f]	NA	35.4 months
									IM	47 vs 41 vs 63	NS			
Lackner et al 2004	Whole				Dako	II – III	Manual	Unclear	IE	70 overall (groups unclear)	NS		NA	81.9 months
									IM	70 overall (groups unclear)	NS			

Takemoto et al 2004	Whole				Dako	II – III	Manual	Arbitrary	IE	17 vs 17 vs 91	NS		31 of 125	Unclear, 4-years quoted
									ST	19 vs 38 vs 68	NS			
Baeten et al 2006	Whole				Dako	I – IV	Manual	Unclear	IE	107 overall (2 groups)	Y	UV[f]	NA	5.8 years
									ST	107 overall (2 groups)	Y	UV[a]		
								Semiquantitative	IM	107 overall (2 groups)	NS			
Galon et al 2006	TMA	4	0.6mm 1mm	2x CT, 2x IM	SP7, Neomarkers	I – III	Electronic (Alphelys)	ROC curve	IE+ST+IM	124 vs 95 vs 30	Y	MV	NA	45.3 months
Deschoolmeester et al 2010	Whole				SPF7, Neomarkers	I – I V	Manual	Semiquantitative	IE	64 vs 141	Y	UV[j]	27 of 215	4.9 years
									ST	82 vs 133	NS			
									IM	150 vs 65	NS			
Nosho et al 2010	TMA	2-4	0.6mm	Representative	F7.2.38, Dako	I – IV	Electronic	Quartiles	IE+ST+IM	727 overall (4 groups)	NS		123 of 753	11.6 years
Katz et al 2013	TMA	3	0.6mm	Liver Met	F7.2.38, Dako	IV	Electronic (Aperio)	Optimal *p*-value	IE+ST	38 vs 149	NS		NA	95 months
Hanke et al 2015	TMA	1	0.6mm	Representative	Dako	II	Manual	Median	IE+ST	132 vs 688	NS		NA	46 months
Vlad et al 2015	TMA	Unclear	Unclear	Unclear	A0452, Dako	II – III	Electronic	Median	IE+ST	22 vs 20	Y	UV[k]	NA	Unclear, 5-years quoted
Chen et al 2016	TMA	2	1mm	Representative	ZSGB-BIO	I – IV	Electronic (TMAJ, JHU)	ROC curve	IE+ST	300 overall (2 groups)	Y	UV[a]	NA	62.9 months
Väyrynen et al 2016	TMA	1-4	3mm	IM + CT		I – IV	Electronic (Image J)	ROC curve	IE	147 overall (2 groups)	NS		11 of 147	51 months

					PS1, Novo-castra				ST	147 overall (2 groups)	NS			
									IM	147 overall (2 groups)	Y	UV		
Schweiger et al 2016	Pulmo-nary Met				SP7, #RM 9107-S1, Thermo Fisher Scientific	IV	Manual	Semiquantitative	IE+ST	27 vs 28	NS		NA	30 months
									IM	32 vs 23	NS			
Li et al 2017	TMA	3	10mm	Random	Thermo	I – IV	Electronic (Image-pro Plus)	Unclear	IE+ST	419 overall (2 groups)	NS		NA	33 months
									IM	419 overall (2 groups)	NS			
Berntsson et al 2017	TMA	2	1mm	Repres-entative	2GV6, Ventana MST	I – IV	Electronic	Optimal *p*-value	IE+ST	338 vs 203	Y	MV[l]	74 of 499	10.5 years
Kim et al 2018	TMA	1	2mm	IM	Dako	I – IV	Electronic (Aperio)	Median	IE	329 vs 327	Y	MV	44 of 488	Unclear, 80-months display
									ST	320 vs 334	Y	UV[h]		
Eriksen et al 2018	Whole				M7254, Dako	II	Electronic	Lower tertile	IE+ST	387 vs 186	Y	MV	173 of 573	7 years
Disease-specific survival														
Laghi et al 2009	Whole				F7.2.38, M7254; Dako	II – III (node-negative)	Electronic	Median	IM	74 vs 74	Y	UV[a]	48 of 286	74 months

Katz et al 2009	TMA (Liver Met)	3	0.6mm	Repres-entative	F7.2.38, Dako	IV	Electronic (Aperio)	Optimal *p*-value	IE+ST	20 vs 142	Y	MV	NA	2 or 10 years
Nosho et al 2010	TMA	2-4	0.6mm	Repres-entative	F7.2.38, Dako	I – IV	Electronic	Quartiles	IE+ST+IM	727 overall (4 groups)	NS		123 of 753	11.6 years
Simpson et al 2010	TMA	3	0.6mm	Random	SP7, NeoMarkers	I – IV	Manual	Mean	IE	234 vs 121	Y	MV	87 of 360	42 months
								Semiquantitative	ST	39 vs 176 vs 141	NS			
Dahlin et al 2011	Whole				Dako	I – IV	Manual	Semiquantitative	IE+ST+IM	116 vs 106 vs 86	Y	UV[m]	72 of 469	50 months
Algars et al 2012	TMA	Unclear	1.2mm	Repres-entative	IgG2a, Acris	II – I V	Manual	Semiquantitative	ST	159 overall (3 groups)	NS		NA	66.2 months
Väyrynen et al 2012	Whole				PS1, Leica	I – IV	Manual vs Electronic	ROC curve	IE+ST+IM	235 overall (2 groups)	Y	MV	NA	60 months
Richards et al 2014	Whole				VP-RM01, Vector	I – III	Manual	Semiquantitative	IE	46 vs 67 vs 134 vs 82	Y	MV	NA	115 months
									ST	53 vs 116 vs 137 vs 23	Y	MV		
									IM	35 vs 95 vs 148 vs 39	Y	MV		
Väyrynen et al 2016	TMA	1-4	3mm	IM + CT	PS1, Novo-castra	I – IV	Electronic (Image J)	ROC curve	IE	147 overall (2 groups)	Y	UV[g]	11 of 147	51 months
									ST	147 overall (2 groups)	NS			
									IM	147 overall (2 groups)	Y	MV[b]		
Nearchou et al 2019	Whole				Fluoresc .A04520	II	Electronic	Optimal *p*-value	IE+ST+IM	114 overall (2 groups)	Y	UV[n]	NA	11.5 years

					1-2, Dako									

[a]no MV given [b]Independent of MSI [c]not independent of FoxP3 or CD45RO [d]not independent of lymph node count/CD8 [e]nil significant on MV [f]Not independent of mast cells/peritumoural inflammatory infiltrate [g]not independent of CD3 in other locations [h]Not independent of CD3/CD8 in IE/ST [j]Not independent of grade/stage [k]not independent of FoxP3 [l]not independent of MSI [m]not independent of MSI/stage/site [n]forward stepwise MV model used but CD3 alone apparently not entered

Table A1.6. Characteristics of studies assessing CD8 in rectal, colon and colorectal cancer

Rectal cancer														
Study	**TMA or Whole section**	**If TMA …**			**Anti-bodies used**	**Cohort assessed (stage)**	**Measu-rement**	**Thresh old**	**Tumour region**	**Groups (overall or high to low)**	**Signifi cant**	**UV or MV**	**MMR**	**Median follow up**
		How many cores?	**Size of cores**	**Choice of core**										
Disease-free survival														
Shinto et al 2014	Biopsy				C8/144B, Dako	II – III	Manual	Median	IE	Unclear	NS		NA	55.1 months
									ST					
	Whole								ST	43 vs 38	Y	MV		
Anitei et al 2014	TMA	4	Uncl ear	2x CT, 2x IM	4B11, Neomarke rs	I – IV	Electron ic (Aperio)	Previous study, (ROC)	IE+ST+ IM	32 vs 40 vs 13	Y	UV[a]	NA	74 months
Teng et al 2015[†]	Biopsy				ZM-0508, Beijing ZGBBC	II – III	Manual	Median	IE+ST	31 vs 31	Y	UV[b]	NA	Unclear, 5 years quoted
Teng et al 2015[†]	Biopsy				ZM-0508, Beijing ZGBBC	II – III	Manual	Mean	ST	75 vs 61	Y	UV[a]	NA	57 months
Posselt et al 2016	Biopsy				Zytomed	I – IV	Electron ic (Biomas)	Median	IE	51 vs 52	NS		NA	3.6 years
									ST	51 vs 51	NS			
	TMA	4	2mm	2x CT, 2x IM					IE	77 vs 76	NS			
									ST	76 vs 76	Y	UV[a]		
McCoy et al 2017	Biopsy				C8/144B, Dako	II – IV	Electron ic (Strata-quest V5)	Median	ST	53 vs 53	NS		NA	78 months
	Biopsy					II – III	Manual	Median	IE	138 vs 137	Y	UV[a]	NA	

Ogura et al 2018	Whole				C8/144B, Nichirei				ST	143 vs 142	NS			57 months
Chen et al 2019	Biopsy				ab4055, Abcam	I – III	Manual	Semiquantitative	ST	36 vs 76	NS		NA	Unclear, 5-years quoted
	TMA	Unclear	2mm	Repres-entative						34 vs 63	Y	UV[c]		
Schollbach et al 2019	Whole				C8/144B, Dako	II – III	Manual	Semiquantitative	IE+ST+IM	42 vs 20 vs 15	NS		NA	Unclear, 5-years quoted
Overall survival														
Anitei et al 2014	TMA	4	0.6mm	2x CT	4B11, Neomarkers	I – IV	Electronic (Aperio)	Previous study, (ROC)	IE+ST+IM	32 vs 40 vs 13	Y	UV[a]	NA	74 months
			1mm	2x IM										
Teng et al 2015[†]	Biopsy				ZM-0508, Beijing ZGBBC	II – III	Manual	Median	IE+ST	31 vs 31	Y	UV[c]	NA	Unclear, 5 years quoted
Teng et al 2015[†]	Biopsy				ZM-0508, Beijing ZGBBC	II – III	Manual	Mean	ST	75 vs 61	Y	UV[a]	NA	57 months
Posselt et al 2016	Biopsy				Zytomed	I – IV	Electronic (Biomas)	Median	IE	51 vs 52	NS		NA	3.6 years
									ST	51 vs 51	NS			
	TMA	4	2mm	2x CT, 2x IM					IE	77 vs 76	NS			
									ST	76 vs 76	NS			
Ogura et al 2018	Biopsy				C8/144B, Nichirei	II – III	Manual	Median	IE	138 vs 137	NS		NA	57 months
	Whole								ST	143 vs 142	NS			
Chen et al 2019	Biopsy				ab4055, Abcam	I – III	Manual	Semiquantitative	ST	36 vs 76	NS		NA	Unclear, 5-years quoted
	TMA	Unclear	2mm	Repres-entative						34 vs 63	NS			

Schollbach et al 2019	Whole				C8/144B, Dako	II – III	Manual	Semiquantitative	IE+ST+IM	42 vs 20 vs 15	Y	MV	NA	Unclear, 5-years quoted
Cancer-specific survival (CSS/DSS)														
Zlobec et al 2008	TMA	1	0.6mm	Representative	C8/144B	I – III	Manual	ROC curve	IE+ST	183 vs 275	Y	MV	31 of 482	51 months
Shinto et al 2014	Biopsy				C8/144B, Dako	II – III	Manual	Median	IE	Unclear	NS		NA	55.1 months
									ST		NS			
	Whole								ST	43 vs 38	Y	MV		
Koelzer et al 2014	Preop biopsy				C8/144B, Dako	I – IV	Manual	75th %ile	IE	17 vs 59	Y	MV	18 of 117	73.5 months
								Median	ST	37 vs 37	Y	MV		
Rosenbaum et al 2016	TMA	2 - 3	2 mm	Repres-entative	IgG2b 4B11, Leica	I – IV (post-nCRT; extra MSI)	Manual	Semiquantitative	IE	15 vs 165	NS		54 of 178	Unclear, 3-years quoted
McCoy et al 2017	Biopsy				C8/144B, Dako	II – IV	Electronic (Strata-quest V5)	Median	ST	53 vs 53	NS		NA	78 months
Colon cancer														
Disease-free survival														
Guidoboni et al 2001	Whole				C8/144B, Dako	II – III (Right-sided)	Electronic	Median	IE	109 overall (2 groups)	Y	MV[d]	47 of 109	78 months
Zlobec et al 2008	TMA	1	0.6mm	Repres-entative	C8/144B, Dako	I – III	Manual	ROC curve	IE+ST	237 overall (2 groups)	Y	MV	0 of 237	Unclear, >5 years display

Correale et al 2010	TMA or biopsy	3	Unclear	Random	C8/144B, Dako	IV	Manual	Semiquantitative	ST	57 overall (groups unclear)	NS		NA	Unclear, 40-months display
Flaherty et al 2016	Whole				SP57, Ventana MST	I – III	Electronic	Continuous	IE+ST	89 overall (2 groups)	Y	UV[a]	NA	33.6 months
								25th %ile	IM	89 overall (2 groups)	Y	UV[a]		
								Continuous	IE+ST+IM	89 overall (2 groups)	Y	MV		
Overall survival														
Guidoboni et al 2001	Whole				C8/144B, Dako	II – III (Right-sided)	Electronic	Median	IE	109 overall (2 groups)	Y	MV[d]	47 of 109	78 months
Correale et al 2010	TMA or biopsy	3	Unclear	Random	C8/144B, Dako	IV	Manual	Semiquantitative	ST	57 overall (groups Unclear)	NS		NA	Unclear, 40-months display
Yoon et al 2012	TMA	3	Unclear	Representative	C8/144B, Dako	II – III	Manual	Median	IE	107 vs 108	Y	UV[e]	22 of 183	8 years
									ST	108 vs 107	Y	MV		
Miller et al 2017	TMA	3	1mm	2xCT, 1xIM	C8/144B, Dako	III	Electronic (Aperio)	Data driven	IE+ST	71 vs 33	NS		18 of 104	82.5 months
									IM	37 vs 18	NS			
Disease-specific survival														
Miller et al 2017	TMA	3	1mm	2xCT, 1xIM	C8/144B, Dako	III	Electronic (Aperio)	Data driven	IE+ST	71 vs 33	NS		18 of 104	82.5 months
									IM	37 vs 18	NS			
Colorectal cancer														
Disease-free survival														
Nagtegaal et al 2001	Whole				Novo-castra	II – IV (160: 40	Manual	Unclear	IE+ST	69 vs 73 vs 58	Y	UV	NA	35.4 months

						Stage II, 40 stage III, 80 stage IV)			IM	86 vs 60	NS			
Prall et al 2004	TMA	3	Unclear	3x IM	C8/144B, Dako	III	Manual	66th %ile	IE+ST	48 vs 104	Y	MV	17 of 152	44 months
Menon et al 2004	Whole				4B11, Novo-castra	II – III	Manual	75th %ile	IE	23 vs 70	NS		6 of 90	6.1 years
								Semiquantitative	ST	16 vs 27 vs 50	NS			
									IM	25 vs 39 vs 25	Y	MV		
Pagès et al 2009††	TMA	4	0.6mm	2x CT,	4B11, Neomarkers	I – II	Electronic (Alphelys)	Optimal *p*-value	IE+ST	239 vs 130	Y	MV	NA	62 months
			1mm	2x IM					IM	252 vs 75	Y	MV		
									IE+ST+ IM	164 vs 92 vs 40	Y	MV		
Suzuki et al 2010	Whole				CM154 BIOCARE	I – IV	Manual	Mean	IE+ST	37 vs 57	NS		NA	Unclear, 8-years display
Deschoolmeester et al 2010	Whole				1A5, Novacastra	I – I V	Manual	Arbitrary	IE	77 vs 138	NS		27 of 215	4.9 years
									ST	132 vs 83	NS			
									IM	144 vs 71	NS			
Tosolini et al 2011††	TMA	2	0.6mm	1x CT	4B11, Neomarkers	I – IV	Electronic	Optimal *p*-value	IE+ST	48 vs 55	Y	MV	NA	Unclear, 5-years quoted
			1mm	1x IM					IM	29 vs 56	Y	MV		
									IE+ST+ IM	19 vs 32 vs 33	Y	MV		
Makkai-popa et al 2013	Whole				Dako	I – III	Electronic	Median	IE+ST	27 overall (2 groups)	NS		NA	Unclear, 36-

							(histo-quest)		IM	27 overall (2 groups)	NS			months display
Katz et al 2013	TMA	3	0.6mm	Liver Met	C8/144B, Dako	IV	Electronic (Aperio)	Optimal *p*-value	IE+ST	36 vs 152	NS		NA	95 months
Kim et al 2015 (training)	TMA	2	2mm	1x CT	Neomarkers	I – IV	Electronic	Median	IE+ST	218 overall (2 groups)	Y	MV	8 of 198	Unclear, 80-months display
			2mm	1x IM					IM	218 overall (2 groups)	Y	MV		
Kim et al 2015 (validation) †††	TMA	1	2mm	IM	Neomarkers	I – IV	Electronic	Median	IM	549 overall (2 groups)	Y	UV[f]	57 of 536	
Lavotshkin et al 2015	Whole				SP57, Ventana MST	I – III	Electronic	Unclear	IE+ST+ IM	35 overall (groups unclear)	NS		NA	46.8 months
Mori et al 2015	Whole				EP1150, GeneTex	I – III	Manual	Median	IE+ST	78 vs 79	Y	MV	9 of 151	20.5 months
Chen et al 2016	TMA	2	1mm	Representative	ZSGB-BIO	I – IV	Electronic (TMAJ, JHU)	ROC curve	IE+ST	300 overall (2 groups)	Y	UV[a]	NA	62.9 months
Väyrynen et al 2016	TMA	1-4	3mm	IM + CT	4B11, Novocastra	I – IV	Electronic (Image J)	ROC curve	IE	147 overall (2 groups)	NS		11 of 147	51 months
									ST	147 overall (2 groups)	NS			
									IM	147 overall (2 groups)	NS			
Schweiger et al 2016	Pulmonary Met				C8/144B, Dako	IV	Manual	Semiquantitative	IE+ST	27 vs 30	NS		NA	30 months
									IM	23 vs 34	NS			

Wei et al 2018	TMA	Unclear	0.6mm	Repres-entative	ZA-0508, ZSGBBIO	I – IV	Manual	Semiquantitative	IE+ST	23 vs 71 vs 328	NS		97 of 354	72 months
Hu et al 2018	TMA	Unclear	0.6mm	Repres-entative	Cell Signalling Tech	I – IV	Manual	ROC curve	IE+ST	276 overall (2 groups)	Y	UV[a]	100 of 276	Unclear, 6-years display
Kim et al 2018†††	TMA	1	2mm	IM	Neomarkers	I – IV	Electronic (Aperio)	Median	IE	388 vs 322	Y	MV	44 of 488	Unclear, 80-months display
									ST	334 vs 320	Y	UV[g]		
Eriksen et al 2018	Whole				M7103, Dako	II	Electronic	Lower tertile	IE+ST	338 vs 185	Y	MV	173 of 573	7 years
Overall survival														
Naito et al 1998	Whole				C8/144B, Dako	I – IV	Manual	Semiquantitative	IE	23 vs 24 vs 28 vs 56	Y	MV	NA	Unclear, 5-years quoted
									ST	Unclear	NS			
									IM	Unclear	NS			
Nagtegaal et al 2001	Whole				Novo-castra	II – IV (160: 40 Stage II, 40 stage III, 80 stage IV)	Manual	Unclear	IE+ST	69 vs 73 vs 58	NS		NA	35.4 months
									IM	86 vs 60	NS			
Takemoto et al 2004	Whole				Dako	II – III	Manual	Arbitrary	IE	17 vs 17 vs 91	NS		31 of 125	60 months
									ST	19 vs 38 vs 68	NS			
Loddenkemper et al 2006	Whole				C8/144B, Dako	I – IV	Manual	Median	IE+ST	40 overall (2 groups)	NS		NA	Unclear
	Whole					I – IV	Manual	Unclear	IE	107 overall	NS		NA	

Baeten et al 2006					Novo-castra					(2 groups)				5.8 years
									ST	107 overall (2 groups)	NS			
								Semiquantitative	IM	107 overall (2 groups)	NS			
Oshikiri et al 2006	Whole				C8/144B, Dako	I – IV	Manual	Unclear	IE+ST	72 vs 74	Y	MV	NA	"Min: 7 years"
Zlobec et al 2008	TMA	1	0.6mm	Repres-entative	C8/144B, Dako	I – II	Manual	ROC	IE+ST	587 overall (2 groups)	Y	MV	0 of 587	Unclear, 5-year quoted
Salama et al 2009	TMA	2	1mm	Random	C8/144B, Dako	II – III	Electronic	Median	IE	967 overall (2 groups)	Y	UV[h]	103 of 956	52.4 months
Pagès et al 2009	TMA	4	0.6mm	2x CT,	4B11, Neomarkers	I – II	Electronic (Alphelys)	Optimal *p*-value	IE+ST	239 vs 130	Y	MV	NA	62 months
			1mm	2x IM					IM	252 vs 75	Y	MV		
									IE+ST+IM	164 vs 92 vs 40	Y	MV		
Kasajima et al 2010	TMA	3	1.5mm	Repres-entative	Dako	I – IV	Manual	Semiquantitative	IE	69 vs 222	Y	UV[j]	NA	38.4 months
									IE+ST	72 vs 219	NS			
Suzuki et al 2010	Whole				CM154 BIOCARE	I – IV	Manual	Mean	IE+ST	37 vs 57	NS		NA	Unclear, 8-years display
Deschoolmeester et al 2010	Whole				1A5, Novo-castra	I – IV	Manual	Arbitrary	IE	77 vs 138	Y	UV[k]	27 of 215	4.9 years
									ST	132 vs 83	NS			
									IM	144 vs 71	NS			
Nosho et al 2010	TMA	2-4	0.6mm	Repres-entative	C8/144B, Dako	I – IV	Electronic	25th %ile	IE+ST+IM	709 overall (4 groups)	Y	UV[l]	123 of 753	11.6 years
Bae et al 2011 (2 cohorts)	TMA	3	2mm	Random	Dako	I – IV (MSIH)	Manual	Unclear	IE	141 vs 28	NS		All 169	38 or 53 months

Katz et al 2013	TMA	3	0.6mm	Liver Met	C8/144B, Dako	IV	Electronic (Aperio)	Optimal *p*-value	IE+ST	36 vs 152	Y	UV[m]	NA	95 months
Lee et al 2013	TMA	1	5mm	1x prim.	C8/144B, Dako	IV	Electronic	Mean	IE+ST	79 overall (2 groups)	Y	UV[n]	NA	39.1 months
				1x met					Met	79 overall (2 groups)	NS			
Hanke et al 2015	TMA	1	0.6mm	Representative	C8/144B, Dako	II	Manual	Median	IE+ST	76 vs 744	NS		NA	46 months
Kim et al 2015 (training)	TMA	2	2mm	1x CT	Neomarkers	I – IV	Electronic	Median	IE+ST	218 overall (2 groups)	NS		8 of 198	Unclear, 80-months display
			2mm	1x IM					IM	218 overall (2 groups)	Y	UV[h]		
Kim et al 2015 (validation) †††	TMA	1	2mm	IM	Neomarkers	I – IV	Electronic	Median	IM	549 overall (2 groups)	Y	UV[h]	57 of 536	
Chen et al 2016	TMA	2	1mm	Representative	ZSGB-BIO	I – IV	Electronic (TMAJ, JHU)	ROC curve	IE+ST	300 overall (2 groups)	NS		NA	62.9 months
Väyrynen et al 2016	TMA	1-4	3mm	IM + CT	4B11, Novocastra	I – IV	Electronic (Image J)	ROC curve	IE	147 overall (2 groups)	NS		11 of 147	51 months
									ST	147 overall (2 groups)	NS			
									IM	147 overall (2 groups)	NS			
Schweiger et al 2016	Pulmonary Met				C8/144B, Dako	IV	Manual	Semiquantitative	IE+ST	27 vs 30	NS		NA	30 months
									IM	23 vs 34	NS			
Li et al 2017	TMA	3	10mm	Random	Thermo	I – IV	Electronic	Unclear	IE+ST	419 overall (2 groups)	NS		NA	33 months

							(Image-pro Plus)		IM	419 overall (2 groups)	NS			
Prizment et al 2017	TMA	3	Unclear	Repres-entative	C8/144B, Dako	I – IV (Women only)	Electronic	Arbitrary	IE+ST	115 vs 208 vs 139 vs 103	Y	MV	104 of 412	8.4 years
Berntsson et al 2017	TMA	2	1mm	Repres-entative	C8/144B, Dako	I – IV	Electronic	Optimal *p*-value	IE+ST	453 vs 77	Y	MV[e]	74 of 499	10.5 years
Sideras et al 2018	Whole liver met				SP-57, Ventana	IV	Electronic (Visio-pharm)	Median	IE+ST	22 vs 23	NS		NA	Unclear, 5-years display
									IM	23 vs 22	NS			
Wei et al 2018	TMA	Unclear	0.6mm	Repres-entative	ZA-0508, ZSGBBIO	I – IV	Manual	Semiquantitative	IE+ST	23 vs 71 vs 328	NS		97 of 354	72 months
Hu et al 2018	TMA	Unclear	0.6mm	Repres-entative	Cell Signalling Tech	I – IV	Manual	ROC curve	IE+ST	276 overall (2 groups)	Y	UV[a]	100 of 276	Unclear, 6-years display
Kim et al 2018[†††]	TMA	1	2mm	IM	Neomarkers	I – IV	Electronic (Aperio)	Median	IE	388 vs 322	Y	UV[g]	44 of 488	Unclear, 80-months display
									ST	334 vs 320	Y	UV[g]		
Eriksen et al 2018	Whole				M7103, Dako	II	Electronic	Lower tertile	IE+ST	338 vs 185	Y	MV	173 of 573	7 years
Nazemalhosseini-Majorad et al 2019	Whole				C8/144B, Dako	I – IV	Manual	Arbitrary	IE	118 vs 86 vs 77	Y	MV	45 of 281	8.4 years
									ST	88 vs 115 vs 78	NS			
Disease-specific survival														
Oberg et al 2002	Whole				C8/144B, Dako	III	Manual	Unclear	ST	17 vs 73	Y	UV[a]	NA	62 months

Funada et al 2003	Whole				Novo-castra	I – IV	Manual	Mean	IM	48 vs 49	Y	UV[a]	NA	Unclear, 60-months display
Prall et al 2004	TMA	3	Unclear	3x IM	C8/144B, Dako	III	Manual	66th %ile	IE+ST	48 vs 104	Y	MV	17 of 152	44 months
Chiba et al 2004	Whole				C8/144B, Dako	I – IV	Manual	Median	IE	194 vs 177	Y	MV	36 of 366	7.7 years
Baker et al 2007	TMA	1 Core	0.6mm	Repres-entative	C8/144B, Dako	I – IV	Manual	ROC curve	IE	554 vs 610	Y	MV[o]	223 of 1420	Unclear, 10-year display
Pagès et al 2009	TMA	4	0.6mm	2x CT,	4B11, Neomar-kers	I – II	Electronic (Alphelys)	Optimal *p*-value	IE+ST	239 vs 130	Y	MV	NA	62 months
			1mm	2x IM					IM	252 vs 75	Y	MV		
									IE+ST+IM	164 vs 92 vs 40	Y	MV		
Lugli et al 2009	Whole (Basel)				C8/144B, Dako	I – IV	Manual	ROC curve	IM	152 vs 113	Y	MV	30 of 125	60 months
Lugli et al 2009	TMA (Athens)	Unclear	0.6mm	?1x IM	C8/144B, Dako	I – IV	Manual	ROC curve	IM	121 vs 69	NS		NA	35 months
Katz et al 2009	TMA (Liver Met)	3	0.6mm	Repres-entative	C8/144B, Dako	IV	Electronic (Aperio)	Optimal *p*-value	IE+ST	26 vs 136	Y	MV	NA	2 or 10 years
Nosho et al 2010	TMA	2-4	0.6mm	Repres-entative	C8/144B, Dako	I – IV	Electronic	25th %ile	IE+ST+IM	709 overall (4 groups)	NS		123 of 753	11.6 years
Richards et al 2014	Whole				M7103, Dako	I – III	Manual	Semiquantitative	IE	37 vs 60 vs 123 vs 107	Y	MV	NA	115 months
									ST	21 vs 61 vs 160 vs 85	Y	UV[p]		
									IM	27 vs 90 vs 134 vs 61	Y	UV[p]		

Ling et al 2014	Whole				C8/144B, Dako	I – IV	Manual	Semiquantitative	IE	22 vs 55 vs 71 vs 109	Y	MV	52 of 253	113 months
									ST	31 vs 108 vs 99 vs 19	Y	UV		
									IM	72 vs 102 vs 63 vs 14	Y	UV		
									IE+ST+ IM	219 vs 128 vs 55	Y	UV		
Väyrynen et al 2016	TMA	1-4	3mm	IM + CT	4B11, Novo-castra	I – IV	Electronic (Image J)	ROC curve	IE	147 overall (2 groups)	NS		11 of 147	51 months
									ST	147 overall (2 groups)	Y	UV[q]		
									IM	147 overall (2 groups)	NS			
Prizment et al 2017	TMA	3	Unclear	Representative	C8/144B, Dako	I – IV (Women only)	Electronic	Arbitrary	IE+ST	115 vs 208 vs 139 vs 103	Y	MV	104 of 412	8.4 years
Matsutani et al 2018 (exploratory)	Whole				C8/144B, Dako	II – III	Manual	Median	IE	73 vs 66	NS		6 of 39	64 months
									ST	70 vs 69	Y	UV[r]		
									IM	70 vs 69	Y	UV[r]		
Matsutani et al 2018 (validation)	Whole				C8/144B, Dako	II – III	Manual	Median from exploratory	IE	42 vs 132	NS		10 of 174	64 months
									ST	149 vs 25	Y	UV[a]		
									IM	138 vs 36	Y	MV		

[a]MV not given [b]not independent of tumour PDL1 [c]not independent of nodal status [d]Independent of MSI [e]not independent of MSI
[f]Not independent of CD45RO/FoxP3 [g]Not independent of CD3/CD8 in IE/ST [h]not independent of FoxP3 and CD45RO
[j]not independent of N-stage/M-stage/age [k]not independent of grade/stage [l]not independent of CD3/CD45RO/FoxP3/MSI/CIMP
[m]not independent of Clinical Risk Score [n]not independent of CD45RO [o]on subgroup analysis, not significant in MMRd
[p]not independent of other markers for T-cells (all highly significant on UV) [q]not independent of CD3/MMR [r]nil significant on MV in this group

[†]Same population, different tumour region assessed [††]Same population, different stage [†††]Same population, different compartment analysed

Table A1.7. Characteristics of studies assessing CD4 in rectal and colorectal cancer

Study	TMA or Whole section	If TMA …			Anti-bodies used	Cohort assessed (stage)	Measu-rement	Threshold	Tumour region	Groups (overall or high to low)	Significant	UV or MV	MMR	Median follow up
		How many cores?	Size of cores	Choice of core										
Rectal cancer														
Disease-free survival														
Teng et al 2015	Preop biopsy				ZA-0519, Beijing ZGBBC	II – III	Manual	Median	IE+ST	31 vs 31	NS		NA	Unclear, 5-years quoted
Overall survival														
Teng et al 2015	Preop biopsy				ZA-0519, Beijing ZGBBC	II – III	Manual	Median	IE+ST	31 vs 31	NS		NA	Unclear, 5-years quoted
Colorectal cancer														
Disease-free survival														
Nagtegaal et al 2001	Whole				Novo-castra	II – IV (160: 40 Stage II, 40 stage III, 80 stage IV)	Manual	Unclear	IE+ST	78 vs 60 vs 66	NS		NA	35.4 months
									IM	70 vs 65 vs 66	NS			
Menon et al 2004	Whole				Clone F6, Novo-castra	II – III	Manual	75th %ile	IE	23 vs 70	NS		6 of 90	6.1 years
								Semiquantitative	ST	23 vs 28 vs 42	NS			
									IM	20 vs 35 vs 36	NS			

Katz et al 2013	TMA	3	0.6mm	Liver Met	R&D Systems	IV	Electronic (Aperio)	Optimal *p*-value	IE+ST	42 vs 145	Y	M V	NA	95 months
Makkai-popa et al 2013	Whole				Dako	I – III	Electronic (histo-quest)	Median	IE+ST	27 overall (2 groups)	NS		NA	Unclear, 36-months display
									IM	27 overall (2 groups)	NS			
Lavotshkin et al 2015	Whole				SP35, Ventana MST	I – III	Electronic	Unclear	IE+ST+IM	35 overall (2 groups)	NS		NA	46.8 months
Chen et al 2016	TMA	2	1mm	Repres-entative	ZSGB-BIO	I – IV	Electronic (TMAJ, JHU)	ROC curve	IE+ST	300 overall (2 groups)	Y	UV [a]	NA	62.9 months
Wei et al 2018	TMA	Unclear	0.6mm	Repres-entative	ZA-0519, ZSGB-BIO	I – IV	Manual	Semiquantitative	IE+ST	19 vs 69 vs 266	NS		97 of 354	72 months
Overall survival														
Nagtegaal et al 2001	Whole				Novo-castra	II – IV (160: 40 Stage II, 40 stage III, 80 stage IV)	Manual	Unclear	IE+ST	78 vs 60 vs 66	NS		NA	35.4 months
									IM	70 vs 65 vs 66	NS			
Kasajima et al 2010	TMA	3	1.5mm	Repres-entative	Dako	I – IV	Manual	Semiquantitative	IE+ST	86 vs 199	Y	UV [b]	NA	38.4 months
Katz et al 2013	TMA	3	0.6mm	Liver Met	R&D Systems	IV	Electronic (Aperio)	Optimal *p*-value	IE+ST	85 vs 77	Y	M V	NA	95 months

Chen & Chen 2014	Whole				Abcam (T-bet)	I – IV	Manual	Unclear	IE+ST+IM	54 vs 48	NS		NA	Unclear, 5-years quoted
Chen et al 2016	TMA	2	1mm	Repres-entative	ZSGB-BIO	I – IV	Electronic (TMAJ, JHU)	ROC curve	IE+ST	300 overall (2 groups)	Y	UV [a]	NA	62.9 months
Li et al 2017	TMA	3	10mm	Random	Thermo	I – IV	Electronic (Image-pro Plus)	Unclear	IE+ST	419 overall (2 groups)	NS		NA	33 months
									IM	419 overall (2 groups)	NS			
Wei et al 2018	TMA	Unclear	0.6mm	Repres-entative	ZA-0519, ZSGB-BIO	I – IV	Manual	Semiquantitative	IE+ST	19 vs 69 vs 266	NS		97 of 354	72 months
Disease-specific survival														
Canna et al 2005	Whole				Vector	II – III	Manual	Tertiles	IE	49 vs 49 vs 49	Y	UV [c]	NA	62 months
Katz et al 2009	TMA (Liver Met)	3	0.6mm	Repres-entative	R&D Systems	IV	Electronic (Aperio)	Optimal *p*-value	IE+ST	85 vs 77	Y	MV	NA	2 or 10 years
Ling et al 2016	Whole				T-bet (H-210; Santa Cruz Biotech)	I – IV	Manual	Semiquantitative	IE+ST	136 vs 147 vs 107	Y	MV	61 of 379	Unclear, 15-years display
Matsutani et al 2018 (exploratory)	Whole				Dako	II – III	Manual	Median	IE	5 vs 134	NS		6 of 39	64 months
									ST	71 vs 68	NS			
									IM	71 vs 68	NS			
	Whole				Dako	II – III	Manual	Median from	IE	21 vs 153	NS			64 months
									ST	40 vs 134	NS			

Matsutani et al 2018 (validation)								exploratory	IM	42 vs 132	NS		10 of 174	

[a]no MV given [b]not independent of N-stage/M-stage/age [c]not independent of age/stage/CRP

Table A1.8. Characteristics of studies assessing CD45RO in rectal, colon and colorectal cancer

Study	TMA or Whole section	If TMA …			Anti-bodies used	Cohort assessed (stage)	Measu-rement	Thresh old	Tumour region	Groups (overall or high to low)	Signi fican t	UV or MV	M M R	Median follow up
		How many cores?	Size of cores	Choice of core										
Rectal cancer														
Disease-free survival														
Wang et al 2015	TMA	Unclea r	1mm	Repres-entative	Novus	II – III (Post nCRT)	Electronic (Alphelys)	Median	IE+ST	91 vs 94	Y	MV	NA	Unclear, 3-years quoted
Overall Survival														
Koelzer et al 2014	Preop biopsy				Abcam	I – IV	Manual	Median	IE	39 vs 37	NS		18 of 117	73.5 Months
									ST	38 vs 38	Y	MV		
Colon cancer														
Disease-free survival														
Lee et al 2010	Whole				Thermo Fisher Scientific	II	Electronic	Mean	IE	87 overall (2 groups)	Y	MV[a]	NA	125 months
									ST	87 overall (2 groups)	Y	UV[b]		
Overall survival														
Lee et al 2010	Whole				Thermo Fisher Scientific	II	Electronic	Mean	IE	87 overall (2 groups)	NS		NA	125 months
									ST	87 overall (2 groups)	Y	UV[b]		
Peng et al 2010	Whole				Zymed	III b	Manual	Arbitrar y, > 75% staining	ST	79 overall (2 groups)	Y	UV[c]	NA	Unclear, 5-years quoted

Colorectal cancer														
Disease-free survival														
Pagès et al 2009[†]	TMA	4	0.6mm	2x CT,	OPD4, Neomarkers	I – II	Electronic (Alphelys)	Optimal *p*-value	IE+ST	302 vs 69	Y	MV	NA	62 months
			1mm	2x IM					IM	242 vs 107	Y	MV		
									IE+ST+IM	188 vs 106 vs 27	Y	MV		
Makkai-popa et al 2013	Whole				Neomarkers	I – III	Electronic (histo-quest)	Median	IE+ST	27 overall (2 groups)	NS		NA	Unclear, 36-months display
									IM	27 overall (2 groups)	NS			
Meshcheryakova et al 2014	Liver met				DB Biotech	IV	Electronic (histo-quest)	Unclear	IM	51 overall (2 groups)	Y	MV	NA	50.2/32.2 months
Kim et al 2015 (training)	TMA	2	2mm	1x CT	Neomarkers	I – IV	Electronic	Median	IE+ST	218 overall (2 groups)	Y	UV[d]	8 of 198	Unclear, 80-months display
			2mm	1x IM					IM	218 overall (2 groups)	Y	MV		
Kim et al 2015 (validation)	TMA	1	2mm	IM	Neomarkers	I – IV	Electronic	Median	IM	549 overall (2 groups)	Y	MV	57 of 536	
Chen et al 2016	TMA	2	1mm	Repres-entative	ZSGB-BIO	I – IV	Electronic (TMAJ, JHU)	ROC curve	IE+ST	300 overall (2 groups)	Y	UV	NA	62.9 months
Schweiger et al 2016	Pulmo-nary Met				UCHL1, #M074201, Dako	IV	Manual	Semiquantitative	IE+ST	32 vs 22	NS		NA	30 months
									IM	39 vs 15	NS			
Overall survival														
Salama et al 2009	TMA	2	1mm	Random	UCHL1, Dako	II – III	Electronic	Median	IE	967 overall (2 groups)	Y	UV[e]	103 of 956	52.4 months
	TMA	4	0.6mm	2x CT,		I – II			IE+ST	302 vs 69	Y	MV	NA	

Pagès et al 2009			1mm	2x IM	OPD4, Neomarkers		Electronic (Alphelys)	Optimal *p*-value	IM	242 vs 107	Y	MV		62 months
									IE+ST+IM	188 vs 106 vs 27	Y	MV		
Nosho et al 2010	TMA	2-4	0.6mm	Repres-entative	UCHL1, Dako	I – IV	Electronic	25th %ile	IE+ST+IM	738 overall (4 groups)	Y	MV[f]	123 of 753	11.6 years
Lee et al 2013	TMA	1	5mm	1x prim.	UCHL1, Dako	IV	Electronic	Mean	IE+ST	79 overall (2 groups)	Y	MV	NA	39.1 months
				1x met					Met	79 overall (2 groups)	Y	UV[g]		
Meshcheryakova et al 2014	Liver met				DB Biotech	IV	Electronic (histo-quest)	Unclear	IM	51 overall (2 groups)	Y	UV	NA	50.2/32.2 months
Kim et al 2015 (training)	TMA	2	2mm	1x CT	Neomarkers	I – IV	Electronic	Median	IE+ST	218 overall (2 groups)	NS		8 of 198	Unclear, 80-months display
			2mm	1x IM					IM	218 overall (2 groups)	Y	MV		
Kim et al 2015 (validation)	TMA	1	2mm	IM	Neomarkers	I – IV	Electronic	Median	IM	549 overall (2 groups)	Y	MV[h]	57 of 536	
Chen et al 2016	TMA	2	1mm	Repres-entative	ZSGB-BIO	I – IV	Electronic (TMAJ, JHU)	ROC curve	IE+ST	300 overall (2 groups)	Y	UV[j]	NA	62.9 months
Schweiger et al 2016	Pulmo-nary Met				UCHL1, #M074201, Dako	IV	Manual	Semiquantitative	IE+ST	32 vs 22	NS		NA	30 months
									IM	39 vs 15	NS			
Disease-specific survival														
Oberg et al 2002	Whole				OPD-4, Dako	III	Manual	Unclear	ST	36 vs 54	Y	UV[j]	NA	62 months
Pagès et al 2009	TMA	4	0.6mm	2x CT,		I – II		Optimal *p*-value	IE+ST	302 vs 69	Y	MV	NA	62 months
			1mm	2x IM					IM	242 vs 107	Y	MV		

					OPD4, Neomarkers		Electronic (Alphelys)		IE+ST+IM	188 vs 106 vs 27	Y	MV		
Katz et al 2009	TMA (Liver Met)	3	0.6mm	Representative	2B11 PD7/26, Dako	IV	Electronic (Aperio)	Optimal *p*-value	IE+ST	148 vs 14	NS		NA	2 or 10 years
Nosho et al 2010	TMA	2-4	0.6mm	Representative	UCHL1, Dako	I – IV	Electronic	25th %ile	IE+ST+IM	738 overall (4 groups)	Y	MV[f]	123 of 753	11.6 years
Richards et al 2014	Whole				M0742, Dako	I – III	Manual	Semiquantitative	IE	31 vs 69 vs 145 vs 85	Y	UV[e]	NA	115 months
									ST	64 vs 116 vs 142 vs 8	Y	UV[e]		
									IM	48 vs 94 vs 141 vs 36	Y	UV[e]		

[a]independent of FoxP3 and CD3 [b]not independent of FoxP3/CD3/VI/NI [c]nil significant on MV [d]not independent of CD8/FoxP3 [e]not independent of FoxP3/CD8/CD3 [f]independent of CD3/CD8/FoxP3/MSI/CIMP [g]not independent of CD45RO in primary tumour [h]independent of FoxP3 and CD8 [j]no MV given

Table A1.9. Characteristics of studies assessing FoxP3 in rectal, colon and colorectal cancer

Rectal cancer															
Study	**TMA or Whole section**	**If TMA …**			**Anti-bodies used**	**Cohort assessed (stage)**	**Measu-rement**	**Threshold**	**Tumour region**	**Groups (overall or high to low)**	**Significant**	**UV or MV**	**Effect**	**MMR**	**Median follow up**
		How many cores?	**Size of cores**	**Choice of core**											
Disease-free survival															
Shinto et al 2014	Preop biopsy				236A/E7, Abcam	II – III	Manual	Median	IE	36 vs 45	NS			NA	55.1 months
									ST	40 vs 41	NS				
	Whole								ST	44 vs 37	NS				
Reimers et al 2014	TMA	3	1mm	Random	236A/E7, Abcam	I – IV	Manual	Median	IE+ST	238 vs 240	Y	MV	+ve	NA	Unclear, 10-years display
Teng et al 2015	Preop biopsy				M3974, Spring Bioscience	II-III	Manual	Median	IE+ST	31 vs 31	NS			NA	Unclear, 5-years quoted
McCoy et al 2015†	TMA	4	1mm	2x CT, 2x “stroma"	236A/E7, Abcam	II-IV (post nCRT)	Electronic (Aperio)	Median	IE	128 overall (2 groups)	Y	UV[a]	-ve	NA	76 months
									ST	128 overall (2 groups)	Y	UV[a]	-ve		
Posselt et al 2016	Biopsy				Zytomed	I – IV (pre- and post-nCRT)	Electronic (Biomas)	Median	IE	51 vs 52	NS			NA	3.6 years
									ST	51 vs 52	NS				
	TMA	4	2mm	2xCT, 2xIM					IE	77 vs 76	NS				
									ST	76 vs 76	NS				

McCoy et al 2017[†]	Biopsy				236A/E7, Abcam	II – IV (pre-nCRT)	Electronic (Strata-quest V5)	Median	IE+ST	53 vs 53	NS			NA	78 months
Overall survival															
Reimers et al 2014	TMA	3	1mm	Random	236A/E7, Abcam	I – IV	Manual	Median	IE+ST	238 vs 240	Y	MV	+ve	NA	Unclear, 10-years display
Teng et al 2015	Preop biopsy				M3974, Spring Bioscience	II-III	Manual	Median	IE+ST	31 vs 31	NS			NA	Unclear, 5-years quoted
Posselt et al 2016	Biopsy				Zytomed	I – IV (pre- and post-nCRT)	Electronic (Biomas)	Median	IE	51 vs 52	Y	UV[b]	+ve	NA	3.6 years
									ST	51 vs 52	Y	UV[b]	+ve		
	TMA	4	2mm	2xCT, 2xIM					IE	77 vs 76	NS				
									ST	76 vs 76	NS				
Disease-specific survival															
Shinto et al 2014	Preop biopsy				236A/E7, Abcam	II – III	Manual	Median	IE	36 vs 45	NS			NA	55.1 months
									ST	40 vs 41	NS				
	Whole								ST	44 vs 37	NS				
McCoy et al 2017	Biopsy				236A/E7, Abcam	II – IV (pre-nCRT)	Electronic (Strata-quest V5)	Median	IE+ST	53 vs 53	NS			NA	78 months
Colon cancer															
Disease-free survival															
Sinicrope et al 2009	TMA	3	Unclear	Repres-entative	ab20034, Abcam	II – III	Manual	1st quartile	IE	101 vs 59	NS			13 of 125	Unclear, 5-years quoted
									ST	118 vs 42	NS				
Lee et al 2010	Whole				PCH1011, eBioscience	II	Electronic	Mean	IE	87 overall	Y	MV	+ve	NA	125 months

										(2 groups)					
									ST	87 overall (2groups)	Y	UV[c]	+ve		
Correale et al 2010	TMA or biopsy	3	Unclear	Random	22510, Abcam	IV	Manual	Semiquantitative	ST	57 overall (groups unclear)	Y	MV	+ve	NA	Unclear, 40-months display
Overall survival															
Sinicrope et al 2009[†]	TMA	3	Unclear	Representative	ab20034, Abcam	II – III	Manual	1st quartile	IE	101 vs 59	NS			13 of 125	Unclear, 5-years quoted
									ST	118 vs 42	NS				
Lee et al 2010	Whole				PCH1011, eBioscience	II	Electronic	Mean	IE	87 overall (2 groups)	Y	UV[c]	+ve	NA	125 months
									ST	87 overall (2groups)	Y	UV[c]	+ve		
Correale et al 2010	TMA or biopsy	3	Unclear	Random	22510, Abcam	IV	Manual	Semiquantitative	ST	57 overall (groups Unclear)	Y	MV	+ve	NA	Unclear, 40-months display
Yoon et al 2012[†]	TMA	3	Unclear	Representative	ab20034, Abcam	II – III	Manual	Median	IE	78 vs 78	Y	MV[d]	+ve	22 of 183	8 years
									ST	78 vs 78	Y	MV[d]	+ve		
									IE+ST	78 vs 78	Unclear				
	TMA	3	1mm			III			IE+ST	87 vs 17	Y	UV[e]	+ve		

Miller et al 2017				2xCT, 1xIM	236A/E7, Abcam		Electronic (Aperio)	Data driven	IM	37 vs 19	NS			18 of 104	82.5 months
Disease-specific survival															
Miller et al 2017	TMA	3	1mm	2xCT, 1xIM	236A/E7, Abcam	III	Electronic (Aperio)	Data driven	IE+ST	87 vs 17	Y	UV[b]	+ve	18 of 104	82.5 months
									IM	37 vs 19	NS				
Markl et al 2017	Whole				SP97, Spring Bio-science	I – IV	Manual	ROC curve	IM	90 vs 46	Y	UV[f]	+ve	21 of 136	55 months
Colorectal cancer															
Disease-free survival															
Suzuki et al 2010	Whole				ab20034, Abcam	I – IV	Manual	Mean	IE+ST	30 vs 64	NS			NA	Unclear, 8-years display
Katz et al 2013	TMA	3	0.6mm	Liver Met	236A/E7, Abcam	IV	Electronic (Aperio)	Optimal *p*-value	IE+ST	26 vs 162	NS			NA	95 months
Zeestraten et al 2013	Whole				ab20034, Abcam	I – III	Manual	Median	IE	36 vs 40	NS			13 of 76	7.3 years
									ST	38 vs 38	NS				
Kim et al 2015 (training)	TMA	2	2mm	1x CT	Abcam	I – IV	Electronic	Median	IE+ST	218 overall (2 groups)	NS			8 of 198	Unclear, 80-months display
			2mm	1x IM					IM	218 overall (2 groups)	NS				
Kim et al 2015 (validation)	TMA	1	2mm	IM	Abcam	I – IV	Electronic	Median	IM	549 overall	Y	UV[g]	+ve	57 of 536	

										(2 groups)					
Lavotshkin et al 2015	Whole				236A/E7, eBioscience s	I – III	Electronic	Unclear	IE+ST+ IM	35 overall (2 groups)	NS				46.8 months
Mori et al 2015	Whole				236A/E, Abcam	I – III	Manual	Median	IE+ST	77 vs 80	NS			9 of 151	20.5 months
Chen et al 2016	TMA	2	1mm	Repres-entative	ZSGB-BIO	I – IV	Electronic (TMAJ, JHU)	ROC curve	IE+ST	300 overall (2 groups)	Y	UV[b]	+ve	NA	62.9 months
Väyrynen et al 2016	TMA	1-4	3mm	IM + CT	236A/E7, AbCam	I – IV	Electronic (Image J)	ROC curve	ST	147 overall (2 groups)	Y	UV[h]	+ve	11 of 147	51 months
									IM	147 overall (2 groups)	Y	UV[h]	+ve		
Schweiger et al 2016	Pulmo -nary Met				206D, #320116, BioLegend	IV	Manual	Semiquan titative	IE+ST	26 vs 27	NS			NA	30 months
									IM	17 vs 36	NS				
Overall survival															
Loddenkem per et al 2006	Whole				PCH101, eBio-science	I – IV	Manual	Median	IE+ST	40 overall (2 groups)	NS			NA	Unclear
Salama et al 2009	TMA	2	1mm	Random	ab20034, Abcam	II – III	Electronic	Median	IE	967 overall	Y	MV	+ve	103 of 956	52.4 months

										(2 groups)					
Suzuki et al 2010	Whole				ab20034, Abcam	I – IV	Manual	Mean	IE+ST	30 vs 64	NS			NA	Unclear, 8-years display
Nosho et al 2010	TMA	2-4	0.6mm	Repres-entative	206D, Biolegend	I – IV	Electronic	25th %ile	IE+ST+ IM	705 overall (4 groups)	Y	UV[j]	+ve	123 of 753	11.6 years
Katz et al 2013	TMA	3	0.6mm	Liver Met	236A/E7, Abcam	IV	Electronic (Aperio)	Optimal *p*-value	IE+ST	26 vs 162	NS			NA	95 months
Lee et al 2013	TMA	1	5mm	1x prim.	PCH1011, Spring-bio-science	IV	Electronic	Mean	IE+ST	79 overall (2 groups)	NS			NA	39.1 months
				1x met					Met	79 overall (2 groups)	Y	UV[k]	+ve		
Zeestraten et al 2013	Whole				ab20034, Abcam	I – III	Manual	Median	IE	36 vs 40	NS			13 of 76	7.3 years
									ST	38 vs 38	NS				
Xu et al 2013	TMA	1	Uncl ear	Random	Abcam	I – IV	Manual	Median	IE+ST	21 vs 69	Y	UV[l]	+ve	NA	65 months
									IE	15 vs 75	Y	MV	-ve		
Chen & Chen 2014	Whole				Abcam	I – IV	Manual	Unclear	IE+ST+ IM	47 vs 55	NS			NA	Unclear, 5-years quoted
Hanke et al 2015	TMA	1	0.6mm	Repres-entative	206D, Biolegend	II	Manual	Median	IE+ST	34 vs 786	Y	MV	+ve	NA	46 months

Vlad et al 2015	TMA	Unclear	Unclear	Unclear	ab20034, Abcam	II – III	Unclear, Possibly electronic	Median	IE+ST	21 vs 21	Y	MV	+ve	NA	Unclear, 5-years quoted
Kim et al 2015 (training)	TMA	2	2mm	1x CT	Abcam	I – IV	Electronic	Median	IE+ST	218 overall (2 groups)	NS			8 of 198	Unclear, 80-months display
			2mm	1x IM					IM	218 overall (2 groups)	NS				
Kim et al 2015 (validation)	TMA	1	2mm	IM	Abcam	I – IV	Electronic	Median	IM	549 overall (2 groups)	Y	UV[g]	+ve	57 of 536	
Wang et al 2015	Whole				ab20034, Abcam	II – III	Manual	Median	IE+ST	181 vs 159	Y	MV	+ve	NA	61.4 months
Chen et al 2016	TMA	2	1mm	Repres-entative	ZSGB-BIO	I – IV	Electronic (TMAJ, JHU)	ROC curve	IE+ST	300 overall (2 groups)	Y	UV[b]	+ve	NA	62.9 months
Väyrynen et al 2016	TMA	1-4	3mm	IM + CT	236A/E7, AbCam	I – IV	Electronic (Image J)	ROC curve	ST	147 overall (2 groups)	Y	UV[h]	+ve	11 of 147	51 months
									IM	147 overall (2 groups)	Y	UV[h]	+ve		
Schweiger et al 2016	Pulmo-nary Met				206D, #320116, BioLegend	IV	Semiquantitative		IE+ST	26 vs 27	NS			NA	30 months
									IM	17 vs 36	NS				

Berntsson et al 2017	TMA	2	1mm	Repres-entative	236A/E7, Abcam	I – IV	Electronic	Optimal *p*-value	IE+ST	300 vs 247	Y	MV[d]	+ve	74 of 499	10.5 years
Sideras et al 2018	Whole liver met				236A/E7, ebio-science	IV	Electronic (visio-pharm)	Median	IE+ST	22 vs 23	NS			NA	Unclear, 5-months display
									IM	23 vs 22	NS				
Disease-specific survival															
Nosho et al 2010	TMA	2-4	0.6mm	Repres-entative	206D, Bio-Legend	I – IV	Electronic	25th %ile	IE+ST+ IM	705 overall (4 groups)	Y	UV[j]	+ve	123 of 753	11.6 years
Frey et al 2010	TMA	1	0.6mm	1x CT	236A/E7, Abcam	I – IV	Manual	ROC curve	IE+ST	267+28 1+73 vs 240+26 0+131	Y	MV[m]	+ve	239 of 143 6	Unclear, 5-years quoted
Ling et al 2014	Whole				236A/E7, AbCam	I – IV	Manual	Semiquan titative	ST	17 vs 83 vs 118 vs 42	Y	MV	+ve	52 of 253	113 months
									IM	15 vs 76 vs 125 vs 39	Y	MV	+ve		
									IE+ST+ IM	125 vs 201 vs 79	Y	MV	+ve		
Richards et al 2014	Whole				ab20034, Abcam	I – III	Manual	Semiquan titative	IE	0 vs 130 vs 122 vs 71	Y	UV[n]	+ve	NA	115 months
									ST	21 vs 61 vs 160 vs 85	Y	UV[n]	+ve		

									IM	0 vs 126 vs 122 vs 63	Y	UV[n]	+ve		
Väyrynen et al 2016	TMA	1-4	3mm	IM + CT	236A/E7, AbCam	I – IV	Electronic (Image J)	ROC curve	ST	147 overall (2 groups)	Y	UV[h]	+ve	11 of 147	51 months
									IM	147 overall (2 groups)	Y	UV[h]	+ve		

[a]Worse outcome from FoxP3, not independent of Dworak/M-stage/PI [b]No MV given [c]not independent of CD3/CD45RO/CD25
[d]not independent of MMR status [e]not independent of Sox2/BRAF/N-stage [f]not independent of T-stage/N-stage
[g]Not independent of CD45RO/CD8 [h]not independent of CD3/MMR [j]not independent of CD3/CD45RO/CD8/MSI/CIMP
[k]not independent of CD45RO, but better prognosis for FoxP3 in met in this study [l]not independent of other pathological features or FoxP3 Tregs within cancer cell nests (negative indicator) [m]not significant in an MMRd subgroup [n]not independent of other markers for T-cells (all highly significant on UV) [†]Same population, different counting method [††]Same population, but different threshold used

Table A1.10. Characteristics of studies assessing Immunoscore in rectal, colon and colorectal cancer

Rectal cancer														
Study	**TMA or Whole section**	**If TMA …**			**Anti-bodies used**	**Cohort assessed (stage)**	**Measurement**	**Threshold**	**Tumour region**	**Groups (overall or high to low)**	**Significant**	**UV or MV**	**MMR**	**Median follow up**
		How many cores?	**Size of cores**	**Choice of core**										
Disease-free survival														
Anitei et al 2014†	TMA	4	0.6mm 1.0mm	2x CT, 2x IM	CD3: 2GV6, Ventana and CD8: C8/144B, Dako	I – IV	Electronic (Aperio) – combined score of **CD3** and **CD8** in 2 tumour regions (CT [IE+ST] and IM)	Optimal *p*-value	(IE+ST) +IM	29 vs 23 vs 21 vs 6 vs 4	Y	MV	NA	74 months
Overall survival														
Anitei et al 2014†	TMA	4	0.6mm 1.0mm	2x CT, 2x IM	CD3: 2GV6, Ventana and CD8: C8/144B, Dako	I – IV	Electronic (Aperio) – combined score of **CD3** and **CD8** in 2 tumour regions (CT [IE+ST] and IM)	Optimal *p*-value	(IE+ST) +IM	29 vs 23 vs 21 vs 6 vs 4	Y	MV	NA	74 months
Colon cancer														
Disease-free survival														
Pagès et al 2018	Whole				CD3: 2GV6, Ventana and CD8: C8/144B, Dako	I – III	Electronic (Aperio) – combined score of **CD3** and **CD8** in 2 tumour regions (CT [IE+ST] and IM)	25% staining	(IE+ST) +IM	708 vs 1271 vs 702	Y	MV	304 of 1562	69 months

Overall survival														
Pagès et al 2018	Whole				CD3: 2GV6, Ventana and CD8: C8/144B, Dako	I – III	Electronic (Aperio) – combined score of **CD3** and **CD8** in 2 tumour regions (CT [IE+ST] and IM)	25% staining	(IE+ST) +IM	708 vs 1271 vs 702	Y	MV	304 of 1562	69 months
Disease-specific survival														
Markl et al 2016	Whole				CD3: MRQ-39, Cell Marque and CD8: SP16, Cell Marque	I-II	Electronic (Image J) – combined score of **CD3** and **CD8** in 2 tumour regions (CT [IE+ST] and IM)	ROC Curve	(IE+ST) +IM (Unable to obtain score for CD8 in CT, so score out of 3; *split 0-1/2/3)*	27 vs 46 vs 73	Y	UV[a]	32 of 170	54 months
Colorectal cancer														
Disease-free survival														
Pagès et al 2009[†]	TMA	4	0.6mm 1.0mm	2x CT, 2x IM	CD8: 4B11 and CD45RO: OPD4, Neomarkers	I – II	Electronic (Alphelys) – combined score of **CD8** and **CD45RO** in 2 tumour regions (CT [IE+ST] and IM)	Optimal *p*-value	(IE+ST) +IM	119 vs 75 vs 76 vs 12	Y	MV	NA	62 months

Mlecnik et al 2011[†] (training)	TMA	2	0.6mm 1.0mm	1x CT 1x IM	CD8: 4B11 and CD45RO: OPD4, Neomarkers	I – III	Electronic (Alphelys) – combined score of **CD8** and **CD45RO** in 2 tumour regions (CT [IE+ST] and IM)	Optimal *p*-value	(IE+ST) +IM	67 vs 46 vs 52 vs 36 vs 14	Y	MV	NA	Unclear, 5-years quoted
Mlecnik et al 2011 (validation)										43 vs 31 vs 30 vs 17 vs 8	Y	MV		
Halama et al 2011	Whole liver met				CD3: PS1, Acris; CD8: 4B11, Novo-castra; and GrzB: 11F1, Novo-castra	IV	Electronic (Visiopharm) – combined score of **CD3** (2points), **CD8** (1 point) and **GrzB** (1point) at metastatic IM.	Unclear	IM (*split 0-2/ 3-4)*	33 overall (2 groups)	Y	UV[b]	0 of 33	Unclear, 6 years display, but nearly all progre-ssed by 30 months
Wirta et al 2017	TMA	4	0.6mm	2CT, 2IM	CD3: PS1, Novo-castra and CD8: SP16, Thermo Scientific	I – IV	Electronic (Aperio) – combined score of **CD3** and **CD8** in 2 tumour regions (CT [IE+ST] and IM)	ROC Curve	(IE+ST) +IM	140 vs 92 vs 86 vs 45 vs 54	Y	MV[c]	80 of 417	6 years
Wang et al 2018	Whole liver met				CD3: ZA0503, ZSGS-BIO, and CD8: ZA0508,	Stage IV CRC	Electronic (Image J) – combined score of **CD3** and **CD8** in 2 tumour regions (CT [IE+ST] and IM). *Immunoscore*	Median	(IE+ST) +IM	90 vs 159	Y	MV	NA	46.4 months

					ZSGS-BIO		*then dichotomised as 0-2/3-4*							
Yomoda et al 2018	Whole				CD3: LN10, Leica and CD8: 4B11, Leica	II – III	Electronic (Image J) – combined score of **CD3** and **CD8** in 2 tumour regions (CT [IE+ST] and IM). *Immunoscore then dichotomised as 0-2/3-4*	Median	(IE+ST) +IM	33 vs 49	Y	MV	NA	Unclear, 5-years display
Overall survival														
Pagès et al 2009†	TMA	4	0.6mm 1.0mm	2x CT, 2x IM	CD8: 4B11 and CD45RO: OPD4, Neomarkers	I – II	Electronic (Alphelys) - combined score of **CD8** and **CD45RO** in 2 tumour regions (CT [IE+ST] and IM)	Optimal *p*-value	(IE+ST) +IM	119 vs 75 vs 76 vs 12	Y	MV	NA	62 months
Mlecnik et al 2011† (training)	TMA	2	0.6mm 1.0mm	1x CT 1x IM	CD8: 4B11 and CD45RO: OPD4, Neomarkers	I – III	Electronic (Alphelys) - combined score of **CD8** and **CD45RO** in 2 tumour regions (CT [IE+ST] and IM)	Optimal *p*-value	(IE+ST) +IM	67 vs 46 vs 52 vs 36 vs 14	Y	MV	NA	Unclear, 5-years quoted
Mlecnik et al 2011 (validation)										43 vs 31 vs 30 vs 17 vs 8	Y	MV		
Halama et al 2011	Whole liver met				CD3: PS1, Acris; CD8: 4B11,	IV	Electronic (Visiopharm) – combined score of **CD3** (2points), **CD8** (1 point) and	Unclear	IM (*split 0-2/ 3-4)*	33 overall (2 groups)	Y	UV[b]	0 of 33	Unclear, 6 years display, but nearly

					Novo-castra; and GrzB: 11F1, Novo-castra		**GrzB** (1point) at metastatic IM.							all progre-ssed by 30 months
Kwak et al 2016	TMA	3	2.0mm	1x CT 1x IM 1x Met	CD3: Dako, CD8: Neomarkers and CD163: Novo-Castra	IV	Electronic (Aperio) – combined score of **CD3** and **CD8** in 2 tumour regions (CT [IE+ST] and IM). Also added additional variables: metastatic IS (ISM: CT [IE+ST]+IM+**Metastases**) and macrophage IS (ISma: CT[IE+ST]+IM+**macrophages**)	Optimal *p*-value.	(IE+ST) +IM (*split 0-2/ 3-4)*	96 vs 96	Y	UV[d]	NA	37.5 months
									(IE+ST) +IM+Met (*split 0-3/ 4-6)*	88 vs 100	Y	MV		
									(IE+ST) +IM+mac (0 for high; 1 for low) (*split 0-3/ 4-6)*	118 vs 75	Y	UV[d]		
Wirta et al 2017	TMA	4	0.6mm	2CT, 2IM	CD3: PS1, Novo-castra and CD8: SP16, Thermo Scientific	I – IV	Electronic (Aperio) – combined score of **CD3** and **CD8** in 2 tumour regions (CT [IE+ST] and IM)	ROC Curve	(IE+ST) +IM	140 vs 92 vs 86 vs 45 vs 54	Y	MV[c]	80 of 417	6 years

Liu et al 2018	Whole				CD3: ZA-0503, and CD8: ZA-0508, ZSGB-BIO	IV	Electronic (Aperio) – combined score as per Kwak et al of **CD3** and **CD8** in 2 tumour regions and metastasis (ISM: CT [IE+ST]+IM+Met)	Same as Kwak	CT [IE+ST] +IM+Met (*split 0-3/ 4-6)*	22 vs 38	NS		NA	Unclear
Wang et al 2018	Whole liver met				CD3: ZA0503, ZSGS-BIO, and CD8: ZA0508, ZSGB-BIO	IV	Electronic (Image J) – combined score of **CD3** and **CD8** in 2 tumour regions (CT [IE+ST] and IM). *Immunoscore then dichotomised as 0-2/3-4*	Median	(IE+ST) +IM	90 vs 159	Y	MV	NA	46.4 months
Yomoda et al 2018	Whole				CD3: LN10, Leica and CD8: 4B11, Leica	II – III	Electronic (Image J) – combined score of **CD3** and **CD8** in 2 tumour regions (CT [IE+ST] and IM). *Immunoscore then dichotomised as 0-2/3-4*	Median	(IE+ST) +IM	33 vs 49	Y	MV	NA	Unclear, 5-years display
Disease-specific survival														
Pagès et al 2009[†]	TMA	4	0.6mm 1.0mm	2x CT, 2x IM	CD8: 4B11 and CD45RO: OPD4,	I – II	Electronic (Alphelys) - combined score of **CD8** and **CD45RO** in 2 tumour regions	Optimal *p*-value	(IE+ST) +IM	119 vs 75 vs 76 vs 12	Y	MV	NA	62 months

					Neomarkers		(CT [IE+ST] and IM)							
Mlecnik et al 2011† (training)	TMA	2	0.6mm 1.0mm	1x CT 1x IM	CD8: 4B11 and CD45RO: OPD4, Neomarkers	I – III	Electronic (Alphelys) - combined score of **CD8** and **CD45RO** in 2 tumour regions (CT [IE+ST] and IM)	Optimal *p*-value	(IE+ST) +IM	67 vs 46 vs 52 vs 36 vs 14	Y	MV	NA	Unclear, 5-years quoted
Mlecnik et al 2011 (validation)										43 vs 31 vs 30 vs 17 vs 8	Y	MV		
Richards et al 2014††	Whole				CD8: M7103 and CD45RO: M0742, Dako	I – III	Manual – combined score of **CD8** and **CD45RO** in 2 tumour regions (CT [IE+ST] and IM)	Semiquantitative	(IE+ST) +IM	58 vs 53 vs 106 vs 91	Y	UV[e]	NA	115 months
Park et al 2016††	Whole				CD3: VP-RM01, Vector and CD8: M7103, Dako	I – III	Manual – combined score of **CD3** and **CD8** in 2 tumour regions (CT [IE+ST] and IM).	Semiquantitative	(IE+ST) +IM	32 vs 43 vs 44 vs 40 vs 87	Not given		30 of 205	150 months
							Immunoscore then split to 3 variables 0-1/2-3/4			32 vs 87 vs 127	Y	MV		
Wirta et al 2017	TMA	4	0.6mm	2CT, 2IM	CD3: PS1, Novo-castra and CD8: SP16, Thermo Scientific	I – IV	Electronic (Aperio) – combined score of **CD3** and **CD8** in 2 tumour regions (CT [IE+ST] and IM)	ROC Curve	(IE+ST) +IM	140 vs 92 vs 86 vs 45 vs 54	Y	MV[c]	80 of 417	6 years

Nearchou et al 2019	Whole				Fluorescent anti-bodies. CD3: A045201-2, Dako and CD8: M7103, Dako	II	Electronic, (High-Plex FL (2.0) and HALO Next-Gen). Combined score of **CD3** and **CD8** in 2 tumour regions (CT [IE+ST] and IM). *Immunoscore then dichotomised as 0-2/3-4*	Optimal *p*-value	(IE+ST) +IM	92 vs 22	Y	UV[b]	NA	Unclear, "max. 11.5"

[a]Unclear regarding MV due to methods [b]no MV given [c]independent of MSI/TNM/etc [d]Not independent of presence of metastases/age/lymphatic invasion [e]Not independent of CD8/CD3 when assessed individually in cancer cell nests and tumour stroma [†]same cohort used for Mlecnik et al 2011 training cohort as for Pagès et al 2009 [††]Same cohort used by both

Table A1.11. Characteristics of studies assessing CD20 in colorectal cancer

Study	TMA or Whole section	If TMA …			Anti-bodies used	Cohort assessed (stage)	Measu-rement	Thresh old	Tumour region	Groups (overall or high to low)	Signifi cant	UV or MV	M M R	Median follow up
		How many cores?	Size of cores	Choice of core										
Disease-free survival														
Bindea et al 2013	TMA	4	0.6mm	2x CT,	L26, Dako	I – IV	Electronic (Alphelys)	ROC curve	IE+ST	107 overall (3 groups)	Y	MV	NA	Unclear, 5-years quoted
			1mm	2x IM										
Meshcheryakova et al 2014	Whole liver met				L26, Thermo Scientific/ E17-P, DB Biotech	IV	Electronic (Tissue-Quest)	Unclear	IM	51 overall (2 groups)	Y	MV	NA	50.2/ 32.2 months
Chen et al 2016	TMA	2	1mm	Repres entativ e	ZSGB-BIO	I – IV	Electronic (TMAJ, JHU)	ROC curve	IE+ST	300 overall (2 groups)	Y	UV[a]	NA	62.9 months
Overall survival														
Baeten et al 2006	Whole				Dako	I – IV	Manual	Unclear	IE	117 overall (2 groups)	NS		NA	5.8 years
									Str	117 overall (2 groups)	NS			
							Semiquan titative	Semiqua ntitative	IM	117 overall (2 groups)	NS			
Kasajima et al 2010	TMA	3	1.5mm	Repres - entativ e	Dako	I – IV	Manual	Semiqua ntitative	IE+ST	3 vs 288	Y	UV[b]	NA	38.4 months
Meshcheryakova et al 2014	Whole liver met				L26, Thermo Scientific/	IV	Electronic (Tissue-Quest)	Unclear	IM	51 overall (2 groups)	Y	UV	NA	50.2/ 32.2 months

					E17-P, DB Biotech									
Chen et al 2016	TMA	2	1mm	Representative	ZSGB-BIO	I – IV	Electronic (TMAJ, JHU)	ROC curve	IE+ST	300 overall (2 groups)	NS		NA	62.9 months
Berntsson et al 2016	TMA	2	1mm	Repres-entative	L6, Ventana	I – IV	Manual	Optimal *p*-value	IE+ST	220 vs 322	Y	MV	76 of 432	5.97 years
Li et al 2017	TMA	3	1cm	Random	Thermo	I – IV	Electronic (Image-pro Plus)	Mean	IE+ST	188 vs 186	Y	UV[a]	NA	33 months
									IM	419 overall (2 groups)	NS			

[a]no MV given [b]not independent of N-stage/M-stage/age (also numbers small in positive group)

Table A1.12. Characteristics of studies assessing CD56/57 in rectal and colorectal cancer

Study	TMA or Whole section	If TMA …			Anti-bodies used	Cohort assessed (stage)	Measu-rement	Threshold	Tumour region	Groups (overall or high to low)	Significant	UV or MV	MMR	Median follow up
		How many cores?	Size of cores	Choice of core										
Rectal cancer														
Overall survival														
Alderdice et al 2017	TMA	2	1mm	Random	CD56: 1B6 Novo-castra	I – III (post nCRT)	Electronic	Arbitrary	IE+ST	48 vs 101	Y	MV[a]	0 of 150	72 months
Colorectal cancer														
Disease-free survival														
Coca et al 1997	Whole				CD57: IOT-10, Immuno-tech SA	I – III	Manual	Arbitrary	IE+ST	25 vs 132	Y	MV	NA	Unclear, 5 years quoted
Nagtegaal et al 2001	Whole				CD56: CAM-16, Becton Dickinson	II – IV (160: 40 Stage II, 40 stage III, 80 stage IV)	Manual	Unclear	IE+ST	73 vs 57	NS		NA	35.4 months
									IM	68 vs 68	NS			
Menon et al 2004	Whole				CD56: 123C3, Zymed	II – III	Manual	75th %ile	IE	23 vs 70	NS		6 of 90	6.1 years
								Semiquantitative	ST	15 vs 29 vs 16	NS			

									IM	12 vs 16 vs 62	NS			
	Whole				CD57: HNK-1, ATCC	II – III	Manual	75th %ile	IE	23 vs 70	NS		6 of 90	6.1 years
								Semiquantitative	ST	30 vs 0 vs 62	NS			
									IM	10 vs 29 vs 48	Y	MV[b]		
Tachibana et al 2005	Whole				C15: Va24[b], Immuno-tech SA	I – IV	Manual	Optimal *p*-value	IE	65 vs 38	Y	MV[c]	NA	5.2 years
Liska et al 2012	Whole				CD57: NK1, Ventana	I – IV	Manual	Unclear	IE+ST	150 overall (2 groups)	NS		NA	Unclear, 5-years display
Overall survival														
Coca et al 1997	Whole				CD57: IOT-10, Immuno-tech SA	I – III	Manual	Arbitrary	IE+ST	25 vs 132	Y	MV	NA	Unclear, 5 years quoted
Nagtegaal et al 2001	Whole				CD56: CAM-16, Becton Dickinson	II – IV (160: 40 Stage II, 40 stage III, 80 stage IV)	Manual	Unclear	IE+ST	73 vs 57	NS		NA	35.4 months
									IM	68 vs 68	NS			
Tachibana et al 2005	Whole				C15: Va24[a], Immu-	I – IV	Manual	Optimal *p*-value	IE	65 vs 38	Y	MV[c]	NA	5.2 years

					notech SA									
Liska et al 2012	Whole				CD57: NK1, Ventana	I – IV	Manual	Unclear	IE+ST	150 overall (2 groups)	Y	UV[d]	NA	Unclear, 5-years display

[a]independent of age/EMVI/TRG/stage [b]independent of CD8 [c]Va24 as a marker of NKT-cells. Independent of N-stage/M-stage/LVI [d]no MV given

Table A1.13. Characteristics of studies assessing CD68/CD163/CD206 in colon and colorectal cancer

Colon cancer															
Study	**TMA or Whole section**	**If TMA …**			**Anti-bodies used**	**Cohort assessed (stage)**	**Measure-ment**	**Threshold**	**Tumour region**	**Groups (overall or high to low)**	**Significant**	**UV or MV**	**Effect (+ve or -ve)**	**MMR**	**Median follow up**
		How many cores?	**Size of cores**	**Choice of core**											
Disease-free survival															
Herrera et al 2013	Whole				**CD163**: 10D6, Novocastra	I – IV	Manual	Lower tertile	ST	157 vs 78	Y	UV[a]	-ve	NA	5 years
Feng et al 2019 (exploratory)	TMA	2	2mm	Random	**CD68**: KP1, Abcam	II	Electronic (Image Pro Plus)	Optimum P-value	ST	148 vs 373	NS			77 of 521	69 months
					CD206: 5C11, Abcam					102 vs 419	Y	UV	-ve		
Feng et al 2019 (validation)	TMA	2	2mm	Random	**CD68**: KP1, Abcam	II	Electronic (Image Pro Plus)	Optimum P-value	ST	75 vs 239	NS			49 of 314	55 months
					CD206: 5C11, Abcam					73 vs 241	Y	UV	-ve		
Overall survival															
Herrera et al 2013	Whole				**CD163**: 10D6, Novocastra	I – IV	Manual	Lower tertile	ST	157 vs 78	Y	UV[a]	-ve	NA	5 years
Feng et al 2019 (exploratory)	TMA	2	2mm	Random	**CD68**: KP1, Abcam	II	Electronic (Image Pro Plus)	Optimum P-value	ST	148 vs 373	NS			77 of 521	69 months

					CD206: 5C11, Abcam					102 vs 419	Y	UV	-ve		
Feng et al 2019 (validation)	TMA	2	2mm	Random	**CD68**: KP1, Abcam	II	Electronic (Image Pro Plus)	Optimum P-value	ST	75 vs 239	NS			49 of 314	55 months
					CD206: 5C11, Abcam					73 vs 241	Y	UV	-ve		
Colorectal cancer															
Disease-free survival															
Makkai-popa et al 2013	Whole				**CD68**: Dako	I – III	Electronic (histo-quest)	Median	IE+ST	27 overall (2 groups)	NS			NA	Unclear, 36-months display
									IM	27 overall (2 groups)	NS				
Meshcheryakova et al 2014	Liver met				**CD68:** Thermo	IV	Electronic (histo-quest)	Unclear	IM	51 overall (2 groups)	NS			NA	50.2/32.2 months
Väyrynen et al 2016	TMA	1-4	3mm	IM + CT	**CD68**: PG-M1, Dako	I – IV	Electronic (Image J)	ROC curve	ST	147 overall (2 groups)	NS			11 of 147	51 months
									IM	147 overall (2 groups)	Y	UV[b]	+ve		
Chen et al 2016	TMA	2	1mm	Repres-entative	**CD68**: Thermo	I – IV	Electronic (TMAJ, JHU)	ROC curve	IE+ST	300 overall (2 groups)	Y	UV[a]	+ve	NA	62.9 months
Shibutani et al 2017	Whole				**CD163**: Leica	II – III	Manual	Median	IM	83 vs 85	Y	MV	-ve	NA	Unclear, 5-years display

Li et al 2018	TMA	Unclear	1.5mm	Repres-entative	**CD68**: Dako	I – IV	Manual	Mean	IE+ST	216 overall (2 groups)	NS			NA	60 months
Kim et al 2018	TMA	1	2mm	IM	**CD68**: Dako	I – IV	Electronic (Aperio)	Median	IE	259 vs 259	Y	MV	-ve	44 of 488	Unclear, 80-months display
									ST	327 vs 327	NS				
					CD163: Leica				IE	258 vs 258	NS				
									ST	326 vs 327	NS				
Overall survival															
Lackner et al 2004	Whole				**CD68**: KP-1, Dako	II – III	Manual	Unclear	IE	70 overall (groups unclear)	NS			NA	81.9 months
									IM		Y	MV	+ve		
Tan et al 2005	Whole				**CD68**: Zhongshan Bio Corp.	I – IV	Manual	Mean	IE+ST	29 vs 31	Y	UV[a]	+ve	NA	Unclear, 5 years quoted
Baeten et al 2006	Whole				**CD68**: Dako	I – IV	Manual	Unclear	IE	117 overall (2 groups)	NS			NA	5.8 years
									IM	117 overall (2 groups)	NS				
Nagorsen et al 2007	Whole				**CD163**: 10D6, Novo-castra	I – IV	Manual	Median	IE	40 overall (2 groups)	NS			NA	Unclear, 5-years display
									ST	40 overall (2 groups)	Y	UV[a]	+ve		
Gulubova et al 2013	Whole				**CD68**: PG-M1, Dako	I – IV	Manual	75th %ile	ST	52 vs 158	NS			NA	39.5 months
									IM	52 vs 158	Y	UV[c]	+ve		

Meshcheryakova et al 2014	Liver met				**CD68:** Thermo	IV	Electronic (histo-quest)	Unclear	IM	51 overall (2 groups)	NS			NA	50.2/32.2 months
Chen et al 2016	TMA	2	1mm	Repres-entative	**CD68**: Thermo	I – IV	Electronic (TMAJ, JHU)	ROC curve	IE+ST	300 overall (2 groups)	Y	UV[c]	+ve	NA	62.9 months
Väyrynen et al 2016	TMA	1-4	3mm	IM + CT	**CD68**: PG-M1, Dako	I – IV	Electronic (Image J)	ROC curve	ST	147 overall (2 groups)	NS			11 of 147	51 months
									IM	147 overall (2 groups)	NS				
Koelzer et al 2016	TMA	8	0.6mm	3xCT; 3xIM; 2x bud	**CD68**: KP1, dako	I – IV	Manual	Mean	IE	38 vs 163	Y	MV	+ve	13 of 203	Unclear, 5-years display
									ST	76 vs 125	Y	MV	+ve		
					CD163: 10D6, Novocastra				ST	53 vs 84	NS				
Shibutani et al 2017	Whole				**CD163**: Leica	II – III	Manual	Median	IM	83 vs 85	Y	MV	-ve	NA	Unclear, 5-years display
Li et al 2017	TMA	3	10mm	Random	**CD68**: Dako	I – IV	Electronic (Image-pro Plus)	Unclear	IE+ST	419 overall (2 groups)	NS			NA	33 months
									IM	199 vs 208	Y	UV[a]	+ve		
Li et al 2018	TMA	Unclear	1.5mm	Repres-entative	**CD68**: Dako	I – IV	Manual	Mean	IE+ST	216 overall (2 groups)	NS			NA	60 months

Ding et al 2018	Whole				**CD163**: Santa Cruz Biotech.	I – IV	Electronic (Image Pro Plus)	ROC-curve	IE+ST	36 vs 37	Y	UV	-ve	NA	Unclear, 30-months display
Kim et al 2018	TMA	1	2mm	IM	**CD68**: Dako	I – IV	Electronic (Aperio)	Median	IE	259 vs 259	Y	UV[d]	-ve	44 of 488	Unclear, 80-months display
									ST	327 vs 327	NS				
					CD163: Leica				IE	258 vs 258	NS				
									ST	326 vs 327	NS				
Disease-specific survival															
Oberg et al 2002	Whole				**CD68**: PG-M1, Dako	III	Manual	Arbitrary	ST	31 vs 59	Y	UV[a]	+ve	NA	62 months
Funada et al 2003	Whole				**CD68**: KP1, Dako	I – IV	Manual	Mean	IM	40 vs 57	NS			NA	Unclear, 5-years quoted
Forssell et al 2007†	Whole				**CD68**: KP-1, Dako	I – IV	Manual	Semiquantitative	IM	61 vs 264 vs 135 vs 18	Y	MV	+ve	NA	Unclear, 10-years display
Zlobec et al 2011	Whole				**CD68**: PG-M1, Dako	I – III	Manual	Quartiles (top 2 combined)	IM	297 overall (3 groups)	Y	MV	+ve	104 of 295	Unclear, 10-years display
Edin et al 2012†	Whole				**CD163**: Novo-castra	I – IV	Manual	Semiquantitative	IM	128 vs 340	Y	UV[e]	+ve	72 of 453	Unclear, >5-years display
Algars et al 2012	TMA	Unclear	1.2mm	Repres-entative	**CD68**: Abcam	II – I V	Manual	Semiquantitative	ST	47 vs 55 vs 43	Y	UV[f]	+ve	NA	66.2 months

Väyrynen et al 2016	TMA	1-4	3mm	IM + CT	**CD68**: PG-M1, Dako	I – IV	Electronic (Image J)	ROC curve	ST	147 overall (2 groups)	NS			11 of 147	51 months
									IM	147 overall (2 groups)	Y	UV [b]	+ve		

[a]No MV given [b]not independent of CD3/MMR/CD83 [c]not independent of VELIPI/mets/age [d]not independent of CD3
[e]Not independent of age/gender/tumour localisation/stage [f]Not independent of stage [†]Same population, different marker

Appendix 2. Supplementary tables comparing the relationship between individual EMT markers and clinicopathological variables

Table A2.1. Associations of clinicopathological variables with EMT marker E-cadherin in stage II-III colorectal cancer (*N*=238)

Clinicopathological characteristics		Membrane E-cadherin				Cytoplasmic E-cadherin				Nuclear E-cadherin				
		Low (n=28) *N* (%)[a]		**High** (n=210) *N* (%)		**Low** (n=140) *N* (%)[a]		**High** (n=98) *N* (%)		**Low** (n=220) *N* (%)		**High** (n=18) *N* (%)		*Pearson* X^2
Age														
≤64	60 (25)	6	(21)	54	(26)	31	(22)	29	(30)	55	(25)	5	(28)	0.82 [m]
65-74	76 (32)	12	(43)	64	(31)	48	(34)	28	(29)	70	(32)	6	(33)	0.39 [c]
≥75	102 (43)	10	(36)	92	(44)	61	(44)	41	(42)	95	(43)	7	(39)	-[b]
Gender														
Female	127 (53)	18	(64)	109	(52)	75	(54)	52	(53)	119	(54)	8	(44)	0.21 [m]
Male	111 (47)	10	(36)	101	(48)	65	(46)	46	(47)	101	(46)	10	(56)	0.94 [c]
Presentation														0.43
Elective	168 (71)	22	(79)	146	(70)	101	(72)	67	(68)	156	(71)	12	(67)	0.32 [m]
Emergency	70 (29)	6	(21)	64	(31)	39	(28)	31	(32)	64	(29)	6	(33)	0.53 [c]
TNM														0.70
I-II (low-risk)	127 (53)	17	(61)	110	(52)	79	(56)	48	(49)	114	(52)	13	(72)	0.41 [m]
II-III (high-risk)	111 (47)	11	(39)	100	(48)	61	(44)	50	(51)	106	(48)	5	(18)	0.25 [c]
T-stage														-[b]
I	1 (1)	0	(0)	1	(1)	1	(1)	0	(0)	1	(1)	0	(0)	-[b]
II	8 (3)	0	(0)	8	(4)	4	(3)	4	(4)	8	(4)	0	(0)	-[b]
III	159 (67)	16	(57)	143	(68)	90	(64)	69	(70)	149	(68)	10	(56)	-[b]
IV	70 (29)	12	(43)	58	(28)	45	(32)	25	(26)	62	(28)	8	(44)	
N-stage														
0	147 (62)	19	(68)	128	(61)	88	(63)	59	(60)	132	(60)	15	(83)	0.70 [m]
I	60 (25)	5	(18)	55	(26)	35	(25)	25	(26)	57	(26)	3	(17)	0.61 [c]
II	31 (13)	4	(14)	27	(13)	17	(12)	14	(14)	31	(14)	0	(0)	-[b]
Site														
Colon	201 (85)	23	(82)	178	(85)	125	(89)	76	(78)	185	(84)	16	(89)	-[b]
Rectum	37 (15)	5	(18)	32	(15)	15	(11)	22	(22)	35	(16)	2	(11)	0.14 [c]
Differentiation														-[b]
Well/mod	206 (87)	20	(71)	186	(89)	117	(84)	89	(91)	188	(86)	18	(100)	0.12 [m]
Poor	32 (13)	8	(29)	24	(11)	23	(16)	9	(9)	32	(15)	0	(0)	0.11 [c]

Venous invasion														-[b]
Absent	156	(66)	17	(61)	139	(66)	96	(69)	60	(61)	143	(65)	13	(72)	0.56[m]
Present	82	(34)	11	(39)	71	(34)	44	(31)	38	(39)	77	(35)	5	(28)	0.24[c]
Tumour budding															-[b]
Present	173	(73)	20	(71)	153	(73)	106	(76)	67	(68)	160	(73)	13	(72)	0.87[m]
Absent	65	(27)	8	(29)	27	(27)	34	(24)	31	(32)	60	(27)	5	(28)	0.21[c]
MMR															-[b]
Proficient	192	(81)	18	(64)	174	(83)	106	(76)	86	(76)	177	(81)	15	(83)	0.19[m]
Deficient	46	(19)	10	(36)	36	(17)	34	(24)	12	(24)	43	(19)	3	(17)	0.21[c]
Tumour perforation															-[b]
No	221	(93)	26	(93)	195	(93)	133	(95)	88	(90)	206	(94)	15	(83)	-[b]
Yes	17	(7)	2	(7)	15	(7)	7	(5)	10	(10)	14	(6)	3	(17)	0.46[c]
Peritoneal involvement															-[b]
No	167	(70)	16	(57)	151	(72)	95	(68)	72	(74)	157	(71)	10	(56)	0.10[m]
Yes	71	(30)	12	(43)	59	(28)	45	(32)	26	(27)	63	(29)	8	(44)	0.35[c]
GMS															0.16
0	61	(26)	9	(32)	52	(25)	38	(27)	23	(24)	55	(25)	6	(33)	0.33[m]
1	133	(56)	12	(43)	121	(58)	76	(54)	57	(58)	122	(56)	11	(61)	0.80[c]
2	44	(18)	7	(25)	18	(18)	26	(19)	18	(19)	43	(20)	1	(6)	-[b]
Modified GPS															
0	83	(53)	10	(56)	73	(53)	45	(50)	38	(59)	77	(53)	6	(67)	-[b]
1	37	(24)	6	(33)	31	(23)	23	(25)	14	(22)	35	(24)	2	(22)	0.45[c]
2	35	(23)	2	(11)	33	(24)	23	(25)	12	(19)	34	(23)	1	(11)	-[b]

[a]percentages rounded to nearest whole number and may not total 100%;

[b]for cells where n<6, *Pearson* X^2 analysis was not performed;

Bold indicates significant result;

[m]membrane locus;

[c]cytoplasmic locus;

[n]nuclear locus

Table A2.2. Associations of clinicopathological variables with EMT marker B-catenin in stage II-III colorectal cancer (*N*=238)

Clinicopathological characteristics		**Membrane B-catenin**				**Cytoplasmic B-catenin**				**Nuclear B-catenin**				
		Low (n=28) *N* (%)[a]		**High** (n=210) *N* (%)		**Low** (n=146) *N* (%)[a]		**High** (n=92) *N* (%)		**Low** (n=96) *N* (%)		**High** (n=142) *N* (%)		*Pearson* X^2
Age														
≤64	60 (25)	5	(18)	55	(26)	41	(28)	19	(21)	26	(27)	34	(24)	0.61 [m]
65-74	76 (32)	11	(39)	65	(31)	50	(34)	26	(28)	30	(31)	46	(32)	0.05 [c]
≥75	102 (43)	12	(43)	90	(43)	55	(38)	47	(51)	40	(42)	62	(44)	0.63
Gender														
Female	127 (53)	17	(61)	110	(52)	84	(58)	43	(47)	58	(60)	69	(49)	0.41 [m]
Male	111 (47)	11	(39)	100	(48)	62	(42)	49	(53)	38	(40)	73	(51)	0.10 [c]
Presentation														0.07
Elective	168 (71)	20	(71)	148	(71)	103	(71)	65	(71)	70	(73)	98	(69)	0.92 [m]
Emergency	70 (29)	8	(29)	62	(30)	43	(30)	27	(29)	26	(27)	44	(31)	0.99 [c]
TNM														0.52
I-II (low-risk)	127 (53)	19	(68)	108	(51)	81	(56)	46	(50)	53	(55)	74	(52)	0.10 [m]
II-III (high-risk)	111 (47)	9	(32)	102	(49)	65	(44)	46	(50)	43	(45)	68	(48)	0.41 [c]
T-stage														0.64
I	1 (1)	0	(0)	1	(1)	1	(1)	0	(0)	1	(1)	0	(0)	- [b]
II	8 (3)	0	(0)	8	(4)	3	(2)	5	(5)	3	(3)	5	(4)	- [b]
III	159 (67)	15	(54)	144	(69)	99	(68)	60	(65)	66	(69)	93	(66)	- [b]
IV	70 (29)	13	(46)	57	(27)	43	(30)	27	(29)	26	(27)	44	(31)	
N-stage														
0	147 (62)	22	(79)	125	(60)	93	(64)	54	(59)	58	(60)	89	(63)	- [b]
I	60 (25)	3	(11)	57	(27)	34	(23)	26	(28)	25	(26)	35	(25)	0.60 [c]
II	31 (13)	3	(11)	28	(13)	19	(13)	12	(13)	13	(14)	18	(13)	0.74
Site														
Colon	201 (85)	24	(86)	177	(84)	126	(86)	75	(82)	87	(91)	114	(80)	- [b]
Rectum	37 (15)	4	(14)	33	(16)	20	(14)	17	(19)	9	(9)	28	(20)	0.32 [c]
Differentiation														**0.03**
Well/mod	206 (87)	22	(79)	184	(88)	121	(83)	85	(92)	76	(79)	130	(92)	0.19 [m]
Poor	32 (13)	6	(21)	26	(12)	25	(17)	7	(8)	20	(21)	12	(9)	**0.03** [c]
Venous invasion														**<0.01**
Absent	156 (66)	21	(75)	135	(64)	95	(65)	61	(66)	63	(66)	93	(66)	0.26 [m]

Present	82	(34)	7	(25)	75	(26)	51	(35)	31	(34)	33	(34)	49	(35)	0.85 [c]
Tumour budding															0.98
Present	173	(73)	17	(61)	156	(74)	107	(73)	66	(72)	71	(74)	102	(72)	0.13 [m]
Absent	65	(27)	11	(39)	54	(26)	39	(27)	26	(28)	25	(26)	40	(28)	0.79 [c]
MMR															0.72
Proficient	192	(81)	16	(57)	176	(84)	107	(73)	85	(92)	61	(64)	131	(92)	**<0.001** [m]
Deficient	46	(19)	12	(43)	34	(16)	39	(27)	7	(8)	35	(36)	11	(8)	**<0.001** [c]
Tumour perforation															**<0.001**
No	221	(93)	25	(89)	196	(93)	137	(94)	84	(91)	91	(95)	130	(92)	-[b]
Yes	17	(7)	3	(11)	14	(7)	9	(6)	8	(9)	5	(5)	12	(8)	0.46 [c]
Peritoneal involvement															-[b]
No	167	(70)	15	(54)	152	(72)	101	(69)	66	(72)	70	(73)	97	(68)	**0.04** [m]
Yes	71	(30)	13	(46)	58	(28)	45	(31)	26	(28)	26	(27)	45	(32)	0.67 [c]
GMS															0.45
0	61	(26)	12	(43)	49	(23)	36	(25)	25	(27)	32	(33)	29	(20)	0.22 [m]
1	133	(56)	10	(36)	123	(59)	81	(56)	52	(57)	50	(52)	83	(59)	0.49 [c]
2	44	(18)	6	(21)	38	(18)	29	(20)	15	(16)	14	(15)	30	(21)	**0.03**
Modified GPS															
0	83	(53)	6	(32)	77	(57)	49	(49)	34	(62)	32	(47)	51	(59)	-[b]
1	37	(24)	10	(53)	27	(20)	25	(25)	12	(22)	19	(28)	18	(21)	0.10 [c]
2	35	(23)	3	(16)	32	(24)	26	(26)	9	(16)	17	(25)	18	(21)	0.23

[a]percentages rounded to nearest whole number and may not total 100%;

[b]for cells where n<6, *Pearson* X^2 analysis was not performed;

Bold indicates significant result;

[m]membrane locus;

[c]cytoplasmic locus;

[n]nuclear locus

Table A2.3. Associations of clinicopathological variables with EMT marker Fascin in stage II-III colorectal cancer (*N*=238)

Clinicopathological characteristics		**Membrane Fascin**			**Cytoplasmic Fascin**			**Nuclear Fascin**			
		Low (n=171) *N* (%)[a]		**High** (n=67) *N* (%)	**Low** (n=31) *N* (%)[a]		**High** (n=207) *N* (%)	**Low** (n=159) *N* (%)		**High** (n=79) *N* (%)	*Pearson* X^2
Age											
≤64	60 (25)	47	(28)	13 (19)	5	(16)	55 (27)	44	(28)	16 (20)	0.15 [m]
65-74	76 (32)	55	(32)	21 (31)	9	(29)	67 (32)	51	(32)	25 (32)	-[b c]
≥75	102 (43)	69	(40)	33 (49)	17	(55)	85 (41)	64	(40)	38 (48)	0.17 [n]
Gender											
Female	127 (53)	89	(52)	38 (57)	17	(55)	110 (53)	81	(51)	46 (58)	0.52 [m]
Male	111 (47)	82	(48)	29 (43)	14	(45)	97 (47)	78	(49)	33 (42)	0.86 [c]
Presentation											0.29 [n]
Elective	168 (71)	121	(71)	47 (70)	20	(65)	148 (72)	112	(70)	56 (71)	0.93 [m]
Emergency	70 (29)	50	(29)	20 (30)	11	(36)	59 (29)	47	(30)	23 (29)	0.43 [c]
TNM											0.94 [n]
I-II (low-risk)	127 (53)	94	(55)	33 (55)	17	(55)	110 (53)	90	(57)	37 (47)	0.43 [m]
II-III (high-risk)	111 (47)	77	(45)	34 (45)	14	(45)	97 (47)	69	(43)	42 (53)	0.86 [c]
T-stage											0.16 [n]
I	1 (1)	1	(1)	0 (0)	0	(0)	1 (1)	1	(1)	0 (0)	-[b m]
II	8 (3)	5	(3)	3 (3)	1	(3)	7 (3)	5	(3)	3 (4)	-[b c]
III	159 (67)	121	(71)	38 (57)	20	(65)	139 (67)	112	(70)	47 (60)	-[b n]
IV	70 (29)	44	(26)	26 (39)	10	(32)	60 (29)	41	(26)	29 (37)	
N-stage											
0	147 (62)	107	(63)	40 (60)	18	(58)	129 (62)	104	(65)	43 (54)	0.35 [m]
I	60 (25)	45	(26)	15 (22)	11	(36)	49 (24)	38	(24)	22 (28)	-[b c]
II	31 (13)	19	(11)	12 (18)	2	(7)	29 (14)	17	(11)	14 (18)	0.07 [n]
Site											
Colon	201 (85)	147	(86)	54 (81)	26	(84)	175 (85)	137	(86)	64 (81)	0.30 [m]
Rectum	37 (15)	24	(14)	13 (19)	5	(16)	32 (16)	22	(14)	15 (19)	-[b c]
Differentiation											0.30 [n]
Well/mod	206 (87)	151	(88)	55 (82)	31	(100)	175 (85)	143	(90)	63 (80)	0.21 [m]
Poor	32 (13)	20	(12)	12 (18)	0	(0)	32 (16)	16	(10)	16 (20)	-[b c]
Venous invasion											**0.03** [n]

Absent	156 (66)	109	(64)	47	(70)	23	(74)	133	(64)	100	(63)	56	(71)	0.35 [m]
Present	82 (34)	62	(36)	20	(30)	8	(26)	74	(36)	59	(37)	23	(29)	0.28 [c]
Tumour budding														0.22 [n]
Present	173 (73)	125	(73)	48	(72)	20	(65)	153	(74)	118	(74)	55	(70)	0.82 [m]
Absent	65 (27)	46	(27)	19	(28)	11	(36)	54	(26)	41	(26)	24	(30)	0.27 [c]
MMR														0.45 [n]
Proficient	192 (81)	139	(81)	53	(79)	24	(77)	168	(81)	129	(81)	63	(80)	0.70 [m]
Deficient	46 (19)	32	(19)	14	(21)	7	(23)	39	(19)	30	(19)	16	(20)	0.62 [c]
Tumour perforation														0.80 [n]
No	221 (93)	160	(94)	61	(91)	30	(97)	191	(92)	150	(94)	71	(90)	0.50 [m]
Yes	17 (7)	11	(6)	6	(9)	1	(3)	16	(8)	9	(6)	8	(10)	-[b c]
Peritoneal involvement														0.21 [n]
No	167 (70)	127	(74)	40	(60)	21	(68)	146	(71)	117	(74)	50	(63)	**0.03** [m]
Yes	71 (30)	44	(26)	27	(40)	10	(32)	61	(30)	42	(26)	29	(37)	0.75 [c]
GMS														0.10 [n]
0	61 (26)	50	(29)	11	(16)	10	(32)	51	(25)	43	(27)	18	(23)	0.21 [m]
1	133 (56)	89	(52)	44	(66)	12	(39)	121	(59)	87	(55)	46	(58)	0.72 [c]
2	44 (18)	32	(19)	12	(18)	9	(29)	35	(17)	29	(18)	15	(19)	0.58 [n]
Modified GPS														
0	83 (53)	65	(56)	18	(47)	9	(39)	74	(56)	59	(54)	24	(53)	0.44 [m]
1	37 (24)	25	(21)	12	(32)	10	(44)	27	(21)	26	(24)	11	(24)	0.56 [c]
2	35 (23)	27	(23)	8	(21)	4	(17)	31	(24)	25	(22)	10	(22)	0.99 [n]

[a]percentages rounded to nearest whole number and may not total 100%;

[b]for cells where n<6, *Pearson* X^2 analysis was not performed;

Bold indicates significant result;

[m]membrane locus;

[c]cytoplasmic locus;

[n]nuclear locus

Table A2.4. Associations of clinicopathological variables with EMT marker Snail in stage II-III colorectal cancer (*N*=238)

Clinicopathological characteristics		**Membrane Snail**				**Cytoplasmic Snail**				**Nuclear Snail**				
		Low (n=46) *N* (%)[a]		**High** (n=192) *N* (%)		**Low** (n=56) *N* (%)[a]		**High** (n=182) *N* (%)		**Low** (n=55) *N* (%)		**High** (n=183) *N* (%)		*Pearson* X^2
Age														
≤64	60 (25)	8	(17)	52	(27)	16	(29)	44	(24)	13	(24)	47	(26)	0.07 [m]
65-74	76 (32)	13	(28)	63	(33)	13	(23)	63	(35)	19	(35)	57	(31)	0.83 [c]
≥75	102 (43)	25	(54)	77	(40)	27	(48)	75	(41)	23	(42)	79	(43)	0.96 [n]
Gender														
Female	127 (53)	24	(52)	103	(54)	33	(59)	94	(52)	26	(47)	101	(55)	0.86 [m]
Male	111 (47)	22	(48)	89	(46)	23	(41)	88	(48)	29	(53)	82	(45)	0.34 [c]
Presentation														0.30 [n]
Elective	168 (71)	30	(65)	138	(72)	39	(70)	129	(71)	38	(69)	130	(71)	0.37 [m]
Emergency	70 (29)	16	(35)	54	(28)	17	(30)	53	(29)	17	(31)	53	(29)	0.86 [c]
TNM														0.78 [n]
I-II (low-risk)	127 (53)	22	(48)	105	(55)	32	(57)	95	(52)	30	(55)	97	(53)	0.40 [m]
II-III (high-risk)	111 (47)	24	(52)	87	(45)	24	(43)	87	(48)	25	(45)	86	(47)	0.52 [c]
T-stage														0.84 [n]
I	1 (1)	0	(0)	1	(1)	0	(0)	1	(1)	0	(0)	1	(1)	-[b m]
II	8 (3)	0	(0)	8	(4)	1	(2)	7	(4)	1	(2)	7	(4)	-[b c]
III	159 (67)	28	(57)	131	(68)	38	(68)	121	(67)	33	(60)	126	(69)	-[b n]
IV	70 (29)	18	(43)	52	(27)	17	(30)	53	(29)	21	(38)	49	(27)	
N-stage														
0	147 (62)	27	(59)	120	(63)	35	(63)	112	(62)	36	(66)	111	(61)	0.31 [m]
I	60 (25)	10	(22)	50	(26)	15	(27)	45	(25)	11	(20)	27	(27)	0.72 [c]
II	31 (13)	9	(20)	22	(12)	6	(11)	25	(14)	8	(15)	13	(13)	0.80 [n]
Site														
Colon	201 (85)	43	(91)	159	(83)	46	(82)	155	(85)	47	(86)	154	(84)	-[b m]
Rectum	37 (15)	4	(9)	33	(17)	10	(18)	27	(15)	8	(15)	29	(16)	0.59 [c]
Differentiation														0.81 [n]
Well/mod	206 (87)	41	(89)	165	(86)	51	(91)	155	(85)	46	(84)	160	(87)	-[b m]
Poor	32 (13)	5	(11)	27	(14)	5	(9)	27	(27)	9	(16)	12	(13)	-[b c]
Venous invasion														0.47 [n]

Absent	156	(66)	30	(65)	126	(66)	37	(66)	119	(65)	40	(73)	116	(63)	0.96 [m]
Present	82	(34)	16	(35)	66	(34)	19	(34)	63	(35)	15	(27)	67	(37)	0.93 [c]
Tumour budding															0.20 [n]
Present	173	(73)	32	(70)	141	(73)	36	(64)	137	(75)	36	(66)	137	(75)	0.60 [m]
Absent	65	(27)	14	(30)	51	(27)	20	(36)	45	(25)	19	(35)	46	(25)	0.11 [c]
MMR															0.17 [n]
Proficient	192	(81)	39	(85)	153	(80)	45	(80)	147	(81)	44	(80)	148	(81)	0.43 [m]
Deficient	46	(19)	7	(15)	39	(20)	11	(20)	35	(19)	11	(20)	35	(19)	0.95 [c]
Tumour perforation															0.89 [n]
No	221	(93)	42	(91)	179	(93)	53	(95)	168	(92)	50	(91)	171	(93)	-[b m]
Yes	17	(7)	4	(9)	13	(7)	3	(5)	14	(8)	5	(9)	12	(7)	-[b c]
Peritoneal involvement															-[b n]
No	167	(70)	28	(61)	139	(72)	39	(70)	128	(70)	34	(62)	133	(73)	0.13 [m]
Yes	71	(30)	18	(39)	53	(28)	17	(30)	54	(30)	21	(38)	50	(27)	0.92 [c]
															0.12 [n]
GMS															
0	61	(26)	11	(24)	50	(26)	17	(30)	44	(24)	13	(24)	48	(26)	0.29 [m]
1	133	(56)	23	(50)	110	(57)	27	(48)	106	(58)	28	(51)	105	(57)	0.82 [c]
2	44	(18)	12	(26)	32	(17)	12	(21)	32	(18)	14	(26)	30	(16)	0.25 [n]
Modified GPS															
0	83	(53)	17	(49)	66	(55)	26	(54)	57	(53)	21	(50)	62	(55)	0.84 [m]
1	37	(24)	11	(31)	26	(22)	15	(31)	22	(21)	13	(31)	24	(21)	0.38 [c]
2	35	(23)	7	(20)	28	(23)	17	(15)	28	(26)	8	(19)	24	(24)	0.99 [n]

[a]percentages rounded to nearest whole number and may not total 100%;

[b]for cells where n<6, *Pearson* X^2 analysis was not performed;

Bold indicates significant result;

[m]membrane locus;

[c]cytoplasmic locus;

[n]nuclear locus

Table A2.5. Associations of clinicopathological variables with EMT marker Zeb1 in stage II-III colorectal cancer (*N*=238)

Clinicopathological characteristics		**Membrane Zeb1**				**Cytoplasmic Zeb1**				**Nuclear Zeb1**				
		Low (n=74) *N* (%)[a]		**High** (n=164) *N* (%)		**Low** (n=195) *N* (%)[a]		**High** (n=43) *N* (%)		**Low** (n=26) *N* (%)		**High** (n=212) *N* (%)		*Pearson* X^2
Age														
≤64	60 (25)	12	(16)	48	(29)	47	(24)	13	(30)	8	(31)	52	(25)	0.12 [m]
65-74	76 (32)	28	(28)	48	(29)	62	(32)	14	(33)	8	(31)	68	(32)	0.34 [c]
≥75	102 (43)	34	(46)	68	(42)	86	(44)	16	(37)	10	(39)	92	(43)	0.51 [n]
Gender														
Female	127 (53)	43	(58)	84	(51)	104	(53)	23	(54)	18	(69)	109	(51)	0.32 [m]
Male	111 (47)	31	(42)	80	(49)	91	(47)	20	(47)	8	(31)	49	(49)	0.99 [c]
Presentation														0.09 [n]
Elective	168 (71)	58	(78)	110	(67)	139	(71)	29	(67)	20	(77)	148	(70)	0.08 [m]
Emergency	70 (29)	16	(32)	54	(33)	56	(29)	14	(33)	6	(33)	64	(30)	0.62 [c]
TNM														0.45 [n]
I-II (low-risk)	127 (53)	43	(58)	84	(51)	99	(51)	28	(65)	13	(50)	114	(54)	0.32 [m]
II-III (high-risk)	111 (47)	31	(42)	80	(49)	96	(49)	15	(35)	13	(50)	98	(46)	0.09 [c]
T-stage														0.72 [n]
I	1 (1)	1	(1)	0	(0)	1	(1)	0	(0)	1	(4)	0	(0)	-[b m]
II	8 (3)	1	(1)	7	(4)	6	(3)	2	(5)	0	(0)	8	(4)	-[b c]
III	159 (67)	48	(65)	111	(68)	128	(66)	31	(72)	16	(62)	143	(68)	-[b n]
IV	70 (29)	24	(32)	46	(28)	60	(31)	10	(23)	9	(35)	31	(29)	
N-stage														
0	147 (62)	50	(68)	97	(59)	118	(61)	29	(67)	16	(62)	131	(62)	0.18 [m]
I	60 (25)	17	(23)	43	(26)	48	(25)	12	(28)	10	(39)	50	(24)	-[b c]
II	31 (13)	7	(10)	24	(15)	29	(15)	2	(5)	0	(0)	31	(15)	-[b n]
Site														
Colon	201 (85)	63	(85)	138	(84)	165	(85)	36	(84)	23	(89)	178	(84)	0.85 [m]
Rectum	37 (15)	11	(15)	26	(16)	30	(15)	7	(16)	3	(11)	34	(16)	0.88 [c]
Differentiation														-[b n]
Well/mod	206 (87)	63	(85)	143	(87)	166	(85)	40	(93)	21	(81)	185	(87)	0.67 [m]
Poor	32 (13)	11	(15)	21	(13)	29	(15)	3	(7)	5	(19)	27	(13)	-[b c]
Venous invasion														-[b n]

Absent	156 (66)	49	(66)	107	(65)	122	(63)	34	(79)	17	(65)	139	(66)	0.88 [m]
Present	82 (34)	25	(34)	57	(35)	73	(37)	9	(21)	9	(35)	73	(34)	**0.04** [c]
Tumour budding														0.99 [n]
Present	173 (73)	54	(73)	119	(73)	140	(72)	33	(77)	15	(58)	158	(75)	0.95 [m]
Absent	65 (27)	20	(27)	45	(27)	55	(28)	10	(23)	11	(42)	54	(25)	0.51 [c]
MMR														0.07 [n]
Proficient	192 (81)	57	(77)	135	(82)	157	(81)	35	(81)	19	(73)	173	(82)	0.34 [m]
Deficient	46 (19)	17	(23)	29	(18)	38	(20)	8	(19)	7	(27)	39	(18)	0.89 [c]
Tumour perforation														0.30 [n]
No	221 (93)	71	(96)	150	(92)	180	(92)	41	(95)	24	(92)	197	(93)	- [b m]
Yes	17 (7)	3	(4)	14	(9)	15	(8)	2	(5)	2	(8)	15	(7)	- [b c]
Peritoneal involvement														- [b n]
No	167 (70)	50	(68)	117	(71)	133	(68)	34	(79)	17	(65)	150	(71)	0.56 [m]
Yes	71 (30)	24	(32)	47	(29)	62	(32)	9	(21)	9	(35)	62	(29)	0.16 [c]
														0.57 [n]
GMS														
0	61 (26)	17	(23)	44	(27)	47	(24)	14	(33)	9	(35)	52	(25)	0.82 [m]
1	133 (56)	43	(58)	90	(55)	109	(56)	24	(56)	14	(54)	119	(56)	- [b c]
2	44 (18)	15	(19)	30	(18)	39	(20)	5	(12)	3	(12)	41	(19)	- [b n]
Modified GPS														
0	83 (53)	31	(56)	52	(52)	67	(53)	16	(57)	11	(52)	72	(54)	0.42 [m]
1	37 (24)	14	(26)	23	(23)	32	(25)	5	(18)	7	(33)	30	(22)	- [b c]
2	35 (23)	10	(18)	25	(25)	28	(22)	7	(25)	3	(14)	32	(24)	- [b n]

[a]percentages rounded to nearest whole number and may not total 100%;

[b]for cells where n<6, *Pearson* X^2 analysis was not performed;

Bold indicates significant result;

[m]membrane locus;

[c]cytoplasmic locus;

[n]nuclear locus

Appendix 3. Tables representing data mined from papers in systematic review of PDCD1/CD274 (Chapter 7) and prognosis in colorectal cancer.

Table A3.1. Summary of tables, cell markers assessed and methodology

First author, year (ref)	Colon (C) Rectal (R) Colorectal (CR)	Section assessed	Threshold	Marker assessed	Anti-body	Blind-ing	Membrane or Cytoplasm	Adjustment variables	Sample size	Surv-ival
Tumour infiltrating lymphocytes										
Berntsson 2018	CR	TMA 1mm	Manual counts of positive TILs (threshold 10% or ROC for absolute PDCD1)	CD274 PDCD1	Cell Signalling (1:200) Abcam (1:50)	Y		Age, Sex, TNM, tumour grade, VI	557	**OS**
Calik 2019	CR	Whole	Manual counts TILs (threshold >5%)	CD274	Master diagnostic (dilutions not specified)	N		Intratumoural CD8, CD8 at IM, CD274 on tumour	157	**DFS**
D'Alterio 2016	CR	CRLM (whole)	Stromal CD274 (>15% threshold) TILs PDCD1 (manual counts, Unclear Threshold)	CD274 PDCD1	Dr Lieping Chen's lab and Spring Bioscience Ventana	Y		Age, sex, number of metastases, KRAS, CXCR4, CXCR7, CD274 mRNA	33	**PFS CSS**
Droeser 2013	CR	TMA 0.6mm	Semiquantitative (strong vs low/absent)	PDCD1	R&D systems (dilutions not specified)	Y		Age, sex, T-stage, N-stage, tumour grade, VI, pattern of invasion, MMR status	423	**OS**
Enkhbat 2018	CR	Whole	>20% T-cells	PDCD1	R&D systems (1:40)	Y		Age, sex, tumour grade, T-stage, TNM (stage III), VI, LI, colonic	116	**DFS OS**

								site (left/right), tumour diameter, PDCD1 tumour		
Hecht 2016	R	Biopsy and TMA 1.6mm	Semiquantitative and percentage max staining cells (median threshold)	CD274	Cell Signalling (1:100)	Y		Age, sex, T-stage, N-stage, M-stage, TNM, Grade	199	**DFS OS**
Ho et al 2019	CR	TMA (unclear)	Semiquantitative in Stroma and counts in intra-epithelial	CD274	Dako (dilutions not specified)	Y		T-stage, TNM, tumour grade, colonic site, LVI, MSI, tumour CD274	238	**OS**
Huang 2018	R	Biopsy	>1% immune cells	PDCD1	Abcam (dilutions not specified)	Y		Age, sex, N-stage, neo-adjuvant response, TRG, cyto-HMGB1	89	**DFS**
Koganemura 2017	CR	Whole	>5% immune cells	CD274	Spring Bioscience (dilutions not specified)	N		Age, sex, colonic site, tumour grade, CD8, adjuvant therapy, CD274 on tumour	235	**DFS**
Ledys 2018	CR	Whole liver met	>5% immune cells	CD274	Roche (pre-diluted)	N	Any	Age, sex, adjuvant therapy, KRAS, BRAF, colonic site, CD8, HLA	114	**OS PFS**
Lee 2016	CR	TMA 0.6mm	>1.43 TILs of >1+ staining intensity	PDCD1	Cell Marque (1:1)	N		Data only shown for MSI subgroup. Age, sex, tumour grade, colonic site, T-stage, N-stage, TNM, TIL score, CLR, CD274.	68 (389)	**RFS**
Lee 2017	CR	TMA 2mm	>5% immune cells	CD274	Cell Signalling (1:50)	N		Age, T-stage, N-stage, M-stage, tumour border, PI, LI, VI, tumour grade	339	**OS**
Lee 2018 (Kyungpook)	C (MSIH)	Whole	Semiquantitative (mod-strong vs weak)	CD274 PDCD1	Abcam (1:100) Cell Marque (1:150)	Y		Age, CEA, tumour grade, TNM, LAG3, IDO1.	89	**DFS OS**
Lee 2018 (Bundang)	CR	TMA 2mm	>5% immune cells	CD274	Dako (ready to use)	Y		TNM, LI, VI, PI, CD274 in tumour	336	**DFS OS**

Li 2016	CR	TMA 0.6mm	Immunoreactivity score >4	PDCD1	Abcam (1:100)	Y		Age, Sex, T-stage, N-stage, M-stage	276	**DFS OS**
Liu 2018 (ZH)	CR	TMA unclear	Electronic counts +ve TILs (present or absent), essentially >1%	CD274	Abcam (dilutions not specified)	N		Age, colonic site, RAS, VI, PI, immunoscore	60	**OS**
Shao 2017	R	Whole	Manual, >10% threshold	CD274	Spring Bioscience (1:100)	Y		Age, sex, T-stage, N-stage, TNM, VI, NI, tumour grade, chemoradiotherapy duration	68	**DFS LRFS OS**
Wei 2018	CRC	TMA 0.6mm	Manual, semiquantitative (>1%)	PDCD1	Spring Biosciences (dilutions not specified)	Y		Age, sex, colonic site, T-stage, N-stage, M-stage, tumour grade, NI, VI, CD4/CD8, MSI	383	**DFS OS**
Tumour cells										
Berntsson 2018	CR	TMA 1mm	>1% positive tumour cells	CD274	Cell Signalling (1:200)	Y	Membrane	Age, Sex, TNM, tumour grade, VI	557	**OS**
Calik 2019	CR	Whole	Manual counts tumour cells (threshold >5%)	CD274	Master diagnostic (dilutions not specified)	N	Any	Intratumoural CD8, CD8 at IM, CD274 on TILs	157	**DFS**
Chen 2019	R	Biopsy and TMA 2mm	>5% positive tumour cells	CD274	Abcam (dilutions not specified)	Y	Membrane	Age, N-stage, Clinical response, TRG, CD8 TILs	112	**DFS**
D'Alterio 2016	CR	CRLM (whole)	Tumour CD274 (>5% threshold)	CD274	Dr Lieping Chen's lab and Spring Bioscience	Y	Membrane	Age, sex, number of metastases, KRAS, CXCR4, CXCR7, CD274 mRNA	33	**PFS CSS**
Droeser 2013	CR	TMA 0.6mm	Semiquantitative (strong vs low/absent)	CD274 CD274	MBL	Y	Unclear	Age, sex, T-stage, N-stage, tumour grade, VI, pattern of invasion, MMR status	384 + 721	**OS**

					Abcam (dilutions not specified)					
Enkhbat 2018	CR	Whole	>50% tumour cells staining	CD274	Abcam (1:100)	Y	Cytoplasm	Age, sex, differentiation, T-stage, TNM (stage III), VI, LI, colonic site (left/right), tumour diameter, CD274 TILs	116	**DFS OS**
Eriksen 2019	C	Whole	>5% positive tumour cells	CD274	Ventana (dilutions not specified)	N	Membrane	Age, sex, T-stage, N-stage, M-stage, colonic site, tumour perforation, LN yield, PI, VI	572	**RFS OS**
Hamada 2017	CR	TMA 0.6mm	Semiquantitative (low 0-1 vs high 2-4)	CD274	eBioscience (1:50)	Y	Cytoplasm + membrane	Age, sex, year of diagnosis, FH, BMI, aspirin/NSAIDs, colonic site, tumour grade, TNM, MSI, etc.	617	**CSS OS**
Hecht 2016	R	Biopsy and TMA 1.6mm	Semiquantitative and percentage max staining cells (median threshold)	CD274	Cell Signalling (1:100)	Y	Unclear	Age, sex, T-stage, N-stage, M-stage, TNM, Grade	199	**DFS OS**
Ho et al 2019	CR	TMA (unclear)	Semiquantitative, weighted histoscore	CD274	Dako (dilutions not specified)	Y	Membrane	T-stage, TNM, tumour grade, colonic site, LVI, MSI, stromal CD274 and intraepithelial TILs CD274	238	**OS**
Koganemura 2017	CR	Whole	>5% tumour cells	CD274	Spring Bioscience (dilutions not specified)	N	Any	Age, sex, colonic site, tumour grade, CD8, adjuvant therapy, CD274 on TILs	235	**DFS**
Ledys 2018	CR	Whole liver met	>5% tumour cells	CD274	Roche (pre-diluted)	N	Any	Age, sex, adjuvant therapy, KRAS, BRAF, colonic site, CD8, HLA	114	**OS PFS**
Lee 2016	CR	TMA 0.6mm	>1% of 2+ intensity	CD274	Cell Signalling (1:250)	N	Membrane	Data only shown for MSI subgroup. Age, sex, tumour grade, colonic	68 (389)	**RFS**

								site, T-stage, N-stage, TNM, TIL score, CLR, PDCD1.		
Lee 2017	CR	TMA 2mm	>5% tumour cells	CD274	Cell Signalling (1:50)	N	Membrane	Age, T-stage, N-stage, M-stage, tumour border, PI, LI, VI, tumour grade	339	**OS**
Lee 2018 (Kyungpook)	C (MSIH)	Whole	>5% tumour cells	CD274	Abcam (1:100)	Y	Membrane	Age, CEA, tumour grade, TNM, LAG3, IDO1.	89	**DFS OS**
Lee 2018 (Bundang)	CR	TMA 2mm	>1% immune cells	CD274	Dako (ready to use)	Y	Membrane	TNM, LI, VI, PI, CD274 in tumour	336	**DFS OS**
Li 2016	CR	TMA 0.6mm	Immunoreactivity score >4	CD274	Abcam (1:50)	Y	Membrane	Age, Sex, T-stage, N-stage, M-stage	276	**DFS OS**
Rosenbaum 2016	R	TMA 2mm	Manual, >5% TPS	CD274	Cell Signalling (1:200)	Y	Membrane	Age, sex, T-stage, N-stage, CD8, BRAF, KRAS, Medullary, MSI	181	**DSS OS**
Saigusa 2016	R	Whole	Manual, semiquantitative (staining intensity 2 or 3)	CD274	Lifespan Biosciences (1:100)	Y	Any	Age, sex, T-stage, N-stage, LI, VI, tumour grade, TRG	100	**RFS OS**
Shao 2017	R	Whole	Manual, >1% threshold	CD274	Spring Bioscience (1:100)	Y	Any	Age, sex, T-stage, N-stage, TNM, VI, NI, tumour grade, XRT duration	68	**DFS LRFS OS**
Shi 201	CR	Whole	Manual, mod-strong vs weak-absent	CD274	Abcam (5mcg/ml)	Y	Any	Age, sex, TNM, colonic site, tumour grade	143	**OS**
Wu 2019	CR	Whole	Manual, >1% threshold	CD274	Abcam (1:50)	Y	Membrane	Age, sex, colonic site, tumour grade, tumour size, T-stage, N-stage, M-stage, TNM, A2aR	204	**OS**

Zhu 2015	CR	Whole	Manual, percentage stained x staining intensity	CD274	Abcam (1:100)	Y	Cytoplasm	Age, Sex, tumour size, colonic site, tumour grade, T-stage, N-stage, M-stage, VI	120	**OS**
Combined scores										
Bae 2018	CR	TMA 3mm	Manual, >50% positive cells	CD274	AnaSpec (1:400)	N	Unclear	T-stage, N-stage, LI, tumour grade, perinodal extension	175	**DFS** **OS**
Hecht 2016	R	Biopsy and TMA 1.6mm	Semiquantitative (low-low for tumour and TILs vs rest)	CD274	Cell Signalling (1:100)	Y	Unclear	Age, sex, T-stage, N-stage, M-stage, TNM, Grade	199	**DFS** **OS**
Miller 2017	C	TMA 1mm	Percentage area of tumour or immune cells (median)	CD274	Cell Signalling (1:100)	Y	Any	T-stage, colonic site, tumour grade, mucin, TILs, BRAF, MSI, other markers	118	**OS** **CSS**
Wei 2018	CR	TMA 0.6mm	Manual, semiquantitative (>1% on TILs and/or >5% on tumour)	CD274	Spring Biosciences (dilutions not specified)	Y	Membrane	Age, sex, colonic site, T-stage, N-stage, M-stage, tumour grade, NI, VI, CD4/CD8, MSI	383	**DFS** **OS**

Table A3.2. Summary of current trials including PD-1 inhibitors and concomitant therapy in colorectal cancer, or solid tumours including colorectal cancers.

Trial status	Trial ID
Anti-PD-1 alone +- NSAID	
Completed	NCT01876511, NCT00729664, NCT00441337, NCT01772004
Active, no longer recruiting	NCT02460198, NCT02227667, NCT01693562, NCT02908906, NCT02054806.
Recruiting	NCT03638297, NCT04118933, NCT03926338, NCT04157985, NCT03755739, NCT03212404, NCT02628067, NCT03150706, NCT03981146, NCT03867799, NCT03435107, NCT03436563
Not yet recruiting	NCT04051450
Anti-PD-1 + standard chemotherapy	
Completed	
Active, no longer recruiting	NCT03904537, NCT02563002, NCT02860546, NCT03174405, NCT02375672, NCT01633970, NCT03414983, NCT02848443, NCT03563157, NCT02870920, NCT02873195, NCT03050814
Recruiting	NCT03374254, NCT03202758, NCT03186326, NCT02842125, NCT03854799, NCT03608046, NCT02997228, NCT03698461, NCT03299660, NCT03827044, NCT04231552, NCT02948348, NCT03921684, NCT03626922, NCT03984578, NCT03844750, NCT03396926, NCT04008030, NCT03388190, NCT03803553, NCT04068610, NCT03376659, NCT03721653,
Not yet recruiting	NCT04194359, NCT04262687, NCT03985891, NCT04072198,
Anti-PD-1 + VEGF/EGF inhibitor	
Completed	NCT02788279, NCT03081494
Active, no longer recruiting	NCT03797326, NCT03174405, NCT02713373, NCT03271047, NCT02873195
Recruiting	NCT04110093, NCT03946917, NCT03647839, NCT03374254, NCT03977090, NCT03912857, NCT03186326, NCT03239145, NCT04171141, NCT03829436, NCT03851614, NCT03608046, NCT03170960, NCT03698461, NCT03657641, NCT03475004, NCT02298959, NCT04126733, NCT03712943, NCT04030260, NCT03373188, NCT02484404, NCT03376659, NCT03475953, NCT02982694, NCT03555149

Not yet recruiting	NCT04262687

Anti-PD-1 + immune stimulant (e.g. vaccine)	
Completed	NCT02981524, NCT02713529, NCT03241173
Active, no longer recruiting	NCT03531632, NCT02757391, NCT02432963, NCT02600949, NCT03473925, NCT02009449, NCT03152565
Recruiting	NCT03206073, NCT04046445, NCT02842125, NCT02636036, NCT03228667, NCT04060342, NCT03775850, NCT03639714, NCT04171141, NCT03953235, NCT03948763, NCT03970382, NCT03761914, NCT02983045, NCT02834052, NCT03724851, NCT01174121, NCT03311334, NCT03329950, NCT03841110, NCT03547999, NCT04208958, NCT03435640, NCT02963831, NCT03376659, NCT03256344, NCT03866239, NCT03289962
Not yet recruiting	NCT03287427, NCT04166383, NCT04195373, NCT04117087
Anti-PD-1 + other checkpoint inhibitor	
Completed	NCT03361228, NCT03241173, NCT02586987, NCT03007407, NCT03005002
Active, no longer recruiting	NCT03274804, NCT02335918, NCT02959437, NCT03168139, NCT03350126, NCT02060188, NCT02178722, NCT03271047, NCT02327078, NCT02888743, NCT01975831, NCT03122509, NCT02870920, NCT03982173
Recruiting	NCT03250832, NCT03642067, NCT03202758, NCT03206073, NCT04157985, NCT03629756, NCT03639714, NCT03507699, NCT02903914, NCT03454451, NCT03953235, NCT02817633, NCT02947165, NCT03517488, NCT02983045, NCT03549000, NCT03126110, NCT03207867, NCT02554812, NCT03799003, NCT04008030, NCT03104439, NCT03184870, NCT03693846, NCT03101475, NCT02740985, NCT02754856, NCT03026140
Not yet recruiting	NCT04258111, NCT04140526, NCT04117087, NCT04145193
Anti-PD-1 + radiotherapy/physical tumour destruction	
Completed	NCT02298946
Active, no longer recruiting	NCT03259867, NCT02437071, NCT02888743, NCT03122509
Recruiting	NCT04001101, NCT03854799, NCT02837263, NCT02992912, NCT03507699, NCT03299660, NCT03058289, NCT04231552, NCT02948348, NCT03921684, NCT03101475, NCT03927898
Not yet recruiting	NCT04108481

Anti-PD-1 + small molecule/kinase inhibitors	
Completed	NCT02777710, NCT01988896, NCT02788279, NCT02586987, NCT03258398, NCT02876224
Active, no longer recruiting	NCT03332498, NCT03631407, NCT02646748, NCT02851004, NCT03377361
Recruiting	NCT03711058, NCT04000529, NCT03829436, NCT03170960, NCT02972034, NCT03791398, NCT04017650, NCT03735628, NCT03428126, NCT03539822, NCT02983578
Not yet recruiting	NCT03601598, NCT04044430, NCT04294160
Anti-PD-1 + cell cycle/DNA metabolism blockade	
Completed	NCT02260440
Active, no longer recruiting	NCT03993626, NCT02437136, NCT02512172, NCT02811497
Recruiting	NCT02890069, NCT03891953, NCT03667716, NCT03454451, NCT03519412, NCT04122625, NCT04256707, NCT03832621, NCT03190174, NCT02484404
Not yet recruiting	NCT03576963
Anti-PD-1 + other	
Completed	
Active, no longer recruiting	
Recruiting	NCT03785210, NCT04014530, NCT03851614, NCT03872947, NCT03658772, NCT03095781, NCT03800602
Not yet recruiting	NCT04119830

Table A3.3. Rectal cancer survival and PDCD1/CD274 expression

PDCD1/CD274 immune cell expression in rectal cancer															
Study	**TMA or Whole section**	**If TMA …**			**Antibodies used**	**Cohort assessed (stage)**	**Measu-rement**	**Threshold**	**Tumour region**	**Groups (overall or high to low)**	**Significant**	**UV or MV**	**Effect**	**MMR**	**Median follow up**
		How many cores?	**Size of cores**	**Choice of core**											
Disease-free survival (DFS/RFS/PFS)															
Hecht et al 2016	Biopsy				**CD274**: E1L3N, Cell Signalling	I – IV	Manual	Semiquantitative	IE+ST	35 vs 68	Y	UV	+ve	NA	Unclear, 5-years display
	TMA	4	1.6mm	2xCT, 2xIM					IE+ST	78 vs 81	NS				
									IM	63 vs 62	Y	MV	+ve		
Shao et al 2017	Whole				**CD274**: Spring Bioscience (1:100)	II – III	Manual	Semiquantitative (>10%)	Unclear	17 vs 51	NS			NA	32.5 months
Huang et al 2018	Pre-nCRT biopsy				**PDCD1**: ab137132, Abcam	II – III	Manual	Present vs absent	IE	29 vs 60	Y	UV[a]	+ve	NA	3 years
Ogura et al 2018	Biopsy				**CD274**: ab205921, Abcam	II-III (pre/post nCRT)	Manual	Semiquantitative	IE	88 vs 187	NS			NA	57 months
									ST	89 vs 192	NS				
	Whole								ST	139 vs 148	NS				
Overall survival															
Hecht et al 2016	Biopsy				**CD274**: E1L3N, Cell Signalling	I – IV	Manual	Semiquantitative	IE+ST	35 vs 68	NS			NA	Unclear, 5-years display
	TMA	4	1.6mm	2xCT, 2xIM					IE+ST	78 vs 81	NS				
									IM	63 vs 62	NS				

Shao et al 2017	Whole				**CD274**: Spring Bioscience (1:100)	II – III	Manual	Semiquantitative (>10%)	Unclear	17 vs 51	NS			NA	32.5 months
Ogura et al 2018	Biopsy				**CD274**: ab205921, Abcam	II – III (pre/post nCRT)	Manual	Semiquantitative	IE	88 vs 187	NS			NA	57 months
									ST	89 vs 192	NS				
	Whole								ST	139 vs 148	NS				
Zhang et al 2019	Biopsy				**CD274**: GeneTech Biotechn.	II – III	Manual	>5% positive TILs	IE+ST	45 vs 64	NS			NA	42 months
	TMA	Unclear	1.8mm	Representative						33 vs 76	NS				
PDCD1/CD274 tumour tissue expression in rectal cancer															
Study	**TMA or Whole section**	**If TMA …**			**Antibodies used**	**Cohort assessed (stage)**	**Measu-rement**	**Threshold**	**Tumour region**	**Groups (overall or high to low)**	**Significant**	**UV or MV**	**Effect**	**MMR**	**Median follow up**
		How many cores?	**Size of cores**	**Choice of core**											
Disease-free survival (DFS/RFS/PFS)															
Saigusa et al 2016	Whole				**CD274**: CD274, 27A2, LifeSpan BioSciences	I – IV	Manual	Semiquantitative	Any	36 vs 54	Y	MV	-ve	NA	46 months
Hecht et al 2016	Biopsy				**CD274**: E1L3N, Cell Signalling	I – IV	Manual	Semiquantitative	Membrane	9 vs 93	NS			NA	Unclear, 5-years display
	TMA	4	1.6mm	2xCT, 2xIM					Membrane (CT)	23 vs 107	NS				

									Membrane (IM)	15 vs 59	NS				
Shao et al 2017	Whole				**CD274**: Spring Bioscience	II – III	Manual	Semiquantitative (>1%)	Any	7 vs 61	NS			NA	32.5 months
Chen et al 2019	Biopsy				**CD274**: ab205921, Abcam	I – III (pre/post nCRT)	Manual	Semiquantitative	Membrane	56 vs 56	Y	MV	+ve	NA	Unclear, 5-years quoted
	TMA	Unclear	2mm	Representative						61 vs 36	Y	MV	+ve		
Overall survival															
Saigusa et al 2016	Whole				**CD274**: CD274, 27A2, LifeSpan BioSciences	I – IV	Manual	Semiquantitative	Any	36 vs 54	Y	MV	-ve	NA	46 months
Hecht et al 2016	Biopsy				**CD274**: E1L3N, Cell Signalling	I – IV	Manual	Semiquantitative	Unclear	9 vs 93	NS			NA	Unclear, 5-years display
	TMA	4	1.6mm	2xCT, 2xIM					Unclear	23 vs 107	NS				
									Unclear	15 vs 59	NS				
Shao et al 2017	Whole				**CD274**: Spring Bioscience	II – III	Manual	Semiquantitative (>1%)	Any	7 vs 61	NS			NA	32.5 months
Chen et al 2019	Biopsy				**CD274**: ab205921, Abcam	I – III (pre/post nCRT)	Manual	Semiquantitative	Membrane	56 vs 56	Y	UV[b]	+ve	NA	Unclear, 5-years quoted
	TMA	Unclear	2mm	Repres-						61 vs 36	Y	UV[b]	+ve		

				entativ e											
Disease-specific survival (DSS/CSS)															
Rosenbaum et al 2016	TMA	2-3	2mm	Repres - entativ e	**CD274**: E1L3N Cell Signalling Technolo gy	I – IV (post nCRT, MSI-enriched)	Manual	Arbitrar y	Membra ne	16 vs 162	NS			54 of 178	Unclea r, 3-years quoted

PDCD1/CD274 combined tumour tissue and immune cell expression in rectal cancer

Study	**TMA or Whole section**	**If TMA …**			**Antibodies used**	**Cohort assessed (stage)**	**Measu-rement**	**Threshold**	**Tumour region**	**Groups (overall or high to low)**	**Significant**	**UV or MV**	**Effect**	**MMR**	**Median follow up**
		How many cores?	**Size of cores**	**Choice of core**											
Disease-free survival (DFS/RFS/PFS)															
Hecht et al 2016	Biopsy				**CD274**: E1L3N, Cell Signalling	I – IV	Manual	Semiquantitative (Low tumour/ TILs vs rest)	Biopsy (Unclear Memb or cyto)	9 vs 93	NS		+ve	NA	Unclear, 5-years display
	TMA	4	1.6mm	2xCT, 2xIM					IT (Unclear Memb or cyto)	23 vs 107	NS				
									IM (Unclear Memb or cyto)	15 vs 59	Y	MV			
Overall survival															
Hecht et al 2016	Biopsy				**CD274**: E1L3N,	I – IV	Manual	Semiquantit-	Biopsy (Unclear	9 vs 93	NS		+ve	NA	Unclear, 5-

					Cell Signalling			ative (Low tumour/ TILs vs rest)	Memb or cyto)						years displa y
	TMA	4	1.6mm	2xCT, 2xIM					IT (Unclear Memb or cyto)	23 vs 107	NS				
									IM (Unclear Memb or cyto)	15 vs 59	Y	M V			

[a]not independent of HMGB1/Nstage [b]No MV given

Table A3.4. Colon cancer survival and PDCD1/CD274 expression

PDCD1/CD274 immune cell expression in colon cancer															
Study	**TMA or Whole section**	**If TMA …**			**Antibodies used**	**Cohort assessed (stage)**	**Measu-rement**	**Thresh old**	**Tumou r region**	**Groups (overall or high to low)**	**Signifi cant**	**UV or M V**	**Effec t**	**MMR**	**Median follow up**
		How many cores?	**Size of cores**	**Choi ce of core**											
Disease-free survival (DFS/RFS/PFS)															
Lee et al 2018	Whole				**CD274**: CD274, Abcam	I – III (**MSIH**)	Manual	Semiqua ntitative	Unclear	56 vs 33	Y	M V	+ve	89 of 89	39 months
					PDCD1: PDCD1, Cell Marque					39 vs 50	NS				
Wyss et al 2019[†]	TMA	6	Uncl ear	CT + IM	**CD274**: SP142, Spring Bioscience	I – IV	Manual	Semiqua ntitative	ST	61 vs 39 vs 27 vs 102	NS			26 of 270	Unclear, 10-months display
Wyss et al 2019[†]	TMA	6	Uncl ear	CT + IM	**PDCD1**: NAT105, Cell Marque	I – IV	Manual	Semiqua ntitative	ST	45 vs 54	Y	UV [a]	+ve	26 of 270	Unclear, 10-months display
Overall survival															
Wyss et al 2019	TMA	6	Uncl ear	CT + IM	**CD274**: SP142, Spring Bioscience	I – IV	Manual	Semiqua ntitative	ST	61 vs 39 vs 27 vs 102	Y	UV [a]	+ve	26 of 270	Unclear, 10-months display

PDCD1/CD274 tumour tissue expression in colon cancer															
Study	**TMA or Whole section**	**If TMA …**			**Antibodies used**	**Cohort assessed (stage)**	**Measu-rement**	**Thresh old**	**Tumou r region**	**Groups (overall or high to low)**	**Signifi cant**	**UV or M V**	**Effec t**	**MMR**	**Median follow up**
		How many cores?	**Size of cores**	**Choi ce of core**											
Disease-free survival (DFS/RFS/PFS)															
Lee et al 2018	Whole				**CD274**: CD274, Abcam	I – III (**MSIH**)	Manual	Semiqua ntitative	Membra ne	89 overall (2 groups)	NS			89 of 89	39 months
Wyss et al 2019	TMA	6	Uncl ear	CT + IM	**CD274**: SP142, Spring Bioscience	I – IV	Manual	Semiqua ntitative	Membra ne	270 overall (2 groups)	NS			26 of 270	Unclear, 10-months display
Eriksen et al 2019	Whole				**CD274:** Ventana	II	Manual	Semiqua ntitative	Membra ne	35 vs 537	NS			172 of 572	6.9 years
PDCD1/CD274 combined tumour tissue and immune cell expression in colon cancer															
Study	**TMA or Whole section**	**If TMA …**			**Antibodies used**	**Cohort assessed (stage)**	**Measu-rement**	**Thresho ld**	**Tumou r region**	**Groups (overall or high to low)**	**Signifi cant**	**UV or M V**	**Effec t**	**MMR**	**Media n follow up**
		How many cores?	**Size of cores**	**Choi ce of core**											
Overall survival															
	TMA	3	1.0mm	2xCT		III	Manual	Median	IE+ST	60 vs 40	NS				

Miller et al 2017				1xIM	**CD274**: E1L3N; Cell Signalling (1:100)				IM	27 vs 29	NS			18 of 104	82.5 months
Disease-specific survival (DSS/CSS)															
Miller et al 2017	TMA	3	1.0mm	2xCT 1xIM	**CD274**: E1L3N; Cell Signalling (1:100)	III	Manual	Median	IE+ST	60 vs 40	NS			18 of 104	82.5 months
									IM	27 vs 29	NS				

[a]no MV given [†]Same study, different antibody/marker

Table A3.5. Colorectal cancer disease-free survival/recurrence-free survival and PDCD1/CD274 expression

PDCD1/CD274 immune cell expression and disease-/recurrence-free survival in colorectal cancer															
Study	**TMA or Whole section**	**If TMA …**			**Antibodies used**	**Cohort assessed (stage)**	**Measu-rement**	**Threshold**	**Tumour region**	**Groups (overall or high to low)**	**Significant**	**UV or MV**	**Effect**	**MMR**	**Median follow up**
		How many cores?	**Size of cores**	**Choice of core**											
Lee et al 2016	TMA	3	0.6mm	Random	**PDCD1**: NAT105, Cell Marque	I – IV (all)	Manual	ROC curve	IE+ST	76 vs 316	Y	UV[a]	+ve	68 of 389	55 months
						I – IV (**MSIH**)				34 vs 34	Y	MV	+ve		
Li et al 2016 (FUSCC)	TMA	Unclear	0.6mm	Random	**PDCD1**: ab137132, Abcam	I – IV	Manual	Arbitrary	IE+ST	106 vs 170	Y	MV	+ve	100 of 276	61 months
Kim et al 2016	TMA	3	2mm	Repres-entative	**CD274**: E1L3N; Cell Signalling	I – IV (**MSIH**)	Manual	Arbitrary	IE+ST	62 vs 146	NS			208 of 208	Unclear, 10-years display
Koganemaru et al 2017	Whole				**CD274**: Spring Bioscience	III	Manual	Arbitrary (>5%)	Unclear	36 vs 199	Y	UV[b]	+ve	NA	52.9 months
Enkhbat et al 2018	Whole				**PDCD1**: AF1086, R&D Systems	II – III	Manual	Arbitrary	ST	39 vs 77	NS		-ve	NA	52 months
Wei et al 2018	TMA	Unclear	0.6mm	Repres-entative	**PDCD1**: SP269, spring bioscience	I – IV	Manual	Semiquantitative	ST	50 vs 304	NS			97 of 354	72 months

Shibutani et al 2018	Whole				**PDCD1**: NAT105, Abcam	II – III	Manual, in TILs in 5HPFs	ROC curve	IM	58 vs 32	NS			NA	Unclear, 5-years display
Wang et al 2018	TMA	2	1mm	Unclear	**CD274**: SP142, Spring Bioscience	II – III	Manual	Unclear	IE+ST	46 vs 208	Y	UV[c]	-ve	NA	42 months
Kollman et al 2018	Met (pulmonary)				**PDCD1**: AF 1086, R&D systems	IV	Manual	Semiquantitative	IE+ST	16 vs 36	NS				30 months
					CD274: E1L3N; Cell Signalling					41 vs 10	NS				
Yomoda et al 2018	Whole				**CD274**: E1L3N; Cell Signalling	II – III	Manual	ROC curve	IE+ST + IM	12 vs 70	NS			NA	Unclear, 5-years display
Calik et al 2018	Whole				**CD274:** Master Diagnostica	I-IV	Manual	Semiquantitative (>5%)	IE+ST	85 vs 72	Y	MV	+ve	NA	52.7 months
Ledys et al 2018	Met (liver)				**CD274**: Roche (pre-diluted)	IV	Manual	Semiquantitative (>5%)	Unclear	34 vs 80	NS			NA	2.9 years
Lee et al 2018	TMA	2	2mm	1x CT 1x IM	**CD274**: Dako (ready to use)	I – IV	Manual	Semiquantitative (>5%)	IE+ST	154 vs 179	Y	MV	+ve	18 of 336	52 months

PDCD1/CD274 tumour tissue expression and disease-/recurrence-free survival in colorectal cancer

Study	TMA or Whole section	If TMA …			Antibodies used	Cohort assessed (stage)	Measu-rement	Threshold	Tumour region	Groups (overall or high to low)	Significant	UV or MV	Effect	MMR	Median follow up
		How many cores?	Size of cores	Choice of core											
Lee et al 2016	TMA	3	0.6mm	Random	**CD274**: E1L3N, Cell Signalling Technology	I – IV (all)	Manual	ROC curve	Cytoplasm	19 vs 375	NS			68 of 389	55 months
						I – IV (**MSIH**)				12 vs 55	Y	MV	-ve		
Li et al 2016 (FUSCC)	TMA	Unclear	0.6mm	Random	**CD274**: ab174838, Abcam	I – IV	Manual	Arbitrary	Unclear	138 vs 138	Y	UV[d]	+ve	100 of 276	61 months
Kim et al 2016	TMA	3	2mm	Repres-entative	**CD274**: E1L3N; Cell Signalling	I – IV (**MSIH**)	Manual	Arbitrary	Membranous-to-cytoplasmic	26 vs 182	NS			208 of 208	Unclear, 10-years display
Koganemaru et al 2017	Whole				**CD274**: Spring Bioscience	III	Manual	Arbitrary (>5%)	Any	19 vs 216	Y	MV	-ve	NA	52.9 months
Enkhbat et al 2018	Whole				**CD274**: ab174838, Abcam	II – III	Manual	Arbitrary	Cytoplasm	52 vs 64	Y	UV[e]	-ve	NA	52 months
Kollman et al 2018	Met (pulmonary)				**PDCD1**: AF 1086, R&D systems	IV	Manual	Semiquantitative	Unclear	35 vs 17	NS			NA	30 months
					CD274: E1L3N; Cell Signalling					36 vs 15	NS				

Calik et al 2018	Whole				**CD274:** Master Diagnostica	I-IV	Manual	Semiquantitative (>5%)	Any	72 vs 85	Y	MV	-ve	NA	52.7 months
Ledys et al 2018	Met (liver)				**CD274**: Roche (pre-diluted)	IV	Manual	Semiquantitative (>5%)	Unclear	5 vs 109	NS			NA	2.9 years
Lee et al 2018	TMA	2	2mm	1x CT 1x IM	**CD274**: Dako (ready to use)	I – IV	Manual	Semiquantitative (>1%)	Membrane	15 vs 321	Y	MV	-ve	18 of 336	52 months

PDCD1/CD274 combined tumour tissue and immune cell expression and disease-/recurrence-free survival in colorectal cancer

Study	**TMA or Whole section**	**If TMA …**			**Antibodies used**	**Cohort assessed (stage)**	**Measu-rement**	**Threshold**	**Tumour region**	**Groups (overall or high to low)**	**Significant**	**UV or MV**	**Effect**	**MMR**	**Median follow up**
		How many cores?	**Size of cores**	**Choice of core**											
Wei et al 2018	TMA	Unclear	0.6mm	Repres-entative	**CD274**: SP142, spring bioscience)	I – IV	Manual	Semiquantitative	ST	162 vs 191	Y	MV	+ve	97 of 354	72 months
Bae et al 2018	TMA	1	3mm	Repres-entative	**CD274:** AnaSpec	I – IV	Manual	50% staining	Uncl	93 vs 82	Y	MV	+ve	NA	88 months

[a]No MV given apart from MMRd subgroup; MMRp tumours alone not significant on UV [b]not independent of tumour CD274/tumour grade/IE CD8 [c]not independent of TNM, p53, Ki67 [d]not independent of age/gender/T-stage/N-stage/M-stage [e]not independent of stage III disease/lymphatic invasion

Table A3.6. Colorectal cancer overall survival and PDCD1/CD274 expression

PDCD1/CD274 immune cell expression and overall survival in colorectal cancer															
Study	**TMA or Whole section**	**If TMA …**			**Antibodies used**	**Cohort assessed (stage)**	**Measu-rement**	**Threshold**	**Tumour region**	**Groups (overall or high to low)**	**Significant**	**UV or MV**	**Effect**	**MMR**	**Median follow up**
		How many cores?	**Size of cores**	**Choice of core**											
Droeser et al 2013	TMA	Unclear	0.6mm	Repres-entative	**CD274**: 27A2, MBL (monoclonal) and ab82059, Abcam (polyclonal)	I – IV (MMR proficient)	Manual	Unclear	ST	11 vs 413	Y	UV[a]	+ve	0 of 424	Unclear, >10-years display
Li et al 2016 (FUSCC)	TMA	Unclear	0.6mm	Random	**PDCD1**: ab137132, Abcam	I – IV	Manual	Arbitrary	IE+ST	106 vs 170	Y	MV	+ve	100 of 276	61 months
Li et al 2016 (TCGA)	TMA	Unclear	0.6mm	Random	**PDCD1**: ab137132, Abcam	I – IV	Manual	Arbitrary	IE+ST	191 vs 165	Y	UV[b]	+ve	113 of 356	13.4 months
Lee et al 2017	TMA	2	2mm	1x CT 1x IM	**CD274**: Cell Signalling Tech	I – IV (MSIH)	Manual	Semiquantitative	IT+ST	107 vs 79	Y	MV	+ve	186 of 186	Unclear, 8-years display
										102 vs 84	Y	MV	+ve		
Lee et al 2017[†]	TMA	2	2mm	1x CT 1x IM	**CD274**: Cell Signalling Tech	I – IV	Manual	Semiquantitative	IE+ST	47 vs 106	Y	MV	+ve	0 of 153	Unclear, 8-years display
										56 vs 97	Y	MV	+ve		
Enkhbat et al 2018	Whole				**PDCD1**: AF1086, R&D Systems	II – III	Manual	Arbitrary	ST	39 vs 77	Y	UV[c]	-ve	NA	52 months

Wei et al 2018	TMA	Unclear	0.6mm	Repres-entative	**PDCD1**: SP269, spring bioscience	I – IV	Manual	Semiquantitative	ST	50 vs 304	NS			97 of 354	72 months
Shibutani et al 2018	Whole				**PDCD1**: NAT105, Abcam	II – III	Manual	ROC curve	IM	39 vs 51	NS			NA	Unclear, 5-years display
Yomoda et al 2018	Whole				**CD274**: E1L3N; Cell Signalling	II – III	Manual	ROC curve	IE+ST+ IM	12 vs 70	NS			NA	Unclear, 5-years display
Liu et al 2018	Whole				**CD274**: ab205921, Abcam	IV	Electronic (Aperio)	Present or absent	IE+ST	26 vs 34	Y	MV	-ve	NA	Unclear, 30-months display
Kollman et al 2018	Met (pulmonary)				**PDCD1**: AF 1086, R&D systems	IV	Manual	Semiquantitative	IE+ST	16 vs 36	Y	UV[d]	-ve	NA	30 months
					CD274: E1L3N; Cell Signalling					41 vs 10	NS				
Berntsson et al 2018	TMA	Unclear	1mm	Repres-entative	**PDCD1:** Abcam	I-IV	Manual	Semiquantitative	IE+ST	298 vs 228	Y	UV[e]	+ve	74 of 575	10 years
					CD274: Cell Signalling					297 vs 239	Y	MV	+ve		
Ledys et al 2018	Met (liver)				**CD274**: Roche (pre-diluted)	IV	Manual	Semiquantitative (>5%)	Unclear	34 vs 80	NS			NA	2.9 years
Lee et al 2018[†]	TMA	2	2mm	1x CT 1x IM	**CD274**: Dako (ready to use)	I – IV	Manual	Semiquantitative (>5%)	IE+ST	154 vs 179	Y	MV	+ve	18 of 336	52 months

Ho et al 2019	TMA	Unclear	Unclear	Unclear	**CD274:** Dako	I-IV	Manual	Present or absent	IE	45 vs 193	Y	UV[f]	+ve	18 of 238	Unclear, 10-years display
								Semiquantitative	Stroma	64 vs 274	Y	MV	+ve		

PDCD1/CD274 tumour tissue expression and overall survival in colorectal cancer

Study	**TMA or Whole section**	**If TMA …**			**Antibodies used**	**Cohort assessed (stage)**	**Measu-rement**	**Threshold**	**Tumour region**	**Groups (overall or high to low)**	**Significant**	**UV or MV**	**Effect**	**MMR**	**Median follow up**
		How many cores?	**Size of cores**	**Choice of core**											
Droeser et al 2013 (training)	TMA	Unclear	0.6mm	Repres-entative	**CD274**: 27A2, MBL (mono-clonal) and ab82059, Abcam (polyclonal)	I – IV (MMR proficient)	Manual	Semiquantitative	Cytoplasm	156 vs 228	Y	UV[g]	+ve	0 of 424	Unclear, >10-years display
Droeser et al 2013 (validation)	TMA	Unclear	0.6mm	Repres-entative	**CD274**: 27A2, MBL (mono-clonal) and ab82059, Abcam (polyclonal)	I – IV (MMR proficient)	Manual	Semiquantitative	Cytoplasm	261 vs 460	Y	UV[g]	+ve	0 of 721	Unclear, >10-years display
Shi et al 2013	Whole				**CD274**: Abcam (5mcg/ml)	I – IV	Manual	Semiquantitative	Any	64 vs 79	Y	MV	+ve	NA	43 months
Zhu et al 2015	Whole				**CD274**: Abcam (1:100)	I – IV	Manual	Arbitrary	Cytoplasm	30 vs 90	Y	UV[h]	-ve	NA	39 months
Li et al 2016 (FUSCC)	TMA	Unclear	0.6mm	Random	**CD274**: ab174838, Abcam	I – IV	Manual	Arbitrary	Unclear	138 vs 138	Y	UV[j]	+ve	100 of 276	61 months

Li et al 2016 (TCGA)	TMA	Unclear	0.6mm	Random	**CD274**: ab174838, Abcam	I – IV	Manual	Arbitrary	Unclear	301 vs 55	Y	UV[k]	+ve	113 of 356	13.4 months
Hamada et al 2017	TMA	2-4	0.6mm	Unclear	**CD274**: CD274, eBioscience	I – IV	Manual	Semiquantitative	Cytoplasm + membrane	384 vs 233	NS			108 of 601	11.5 years
Lee et al 2017[†]	TMA	2	2mm	1x CT 1x IM	**CD274**: Cell Signalling Tech	I – IV (MSIH)	Manual	Semiquantitative	Membrane	43 vs 143	NS			186 of 186	Unclear, 8-years display
										47 vs 141	NS				
Enkhbat et al 2018	Whole				**CD274**: ab174838, Abcam	II – III	Manual	Arbitrary	Cytoplasm	52 vs 64	Y	MV	-ve	NA	52 months
Kollman et al 2018	Met (pulmonary)				**PDCD1**: AF 1086, R&D systems	IV	Manual	Semiquantitative	Unclear	35 vs 17	NS			NA	30 months
					CD274: E1L3N; Cell Signalling					36 vs 15	NS				
Berntsson et al 2018	TMA	Unclear	1mm	Representative	**PDCD1:** Abcam	I-IV	Manual	Semiquantitative	Membrane	298 vs 228	NS			74 of 575	10 years
Ledys et al 2018	Met (liver)				**CD274**: Roche (pre-diluted)	IV	Manual	Semiquantitative (>5%)	Unclear	5 vs 109	NS			NA	2.9 years
Lee et al 2018[†]	TMA	2	2mm	1x CT 1x IM	**CD274**: Dako (ready to use)	I – IV	Manual	Semiquanta	IE+ST	15 vs 321	Y	MV	-ve	18 of 336	52 months

								tive (>1%)							
Ho et al 2019	TMA	Unclear	Unclear	Unclear	**CD274**: Dako (dilutions not specified)	I – IV	Manual	Weighted histo-score (threshold 10)	Membrane	13 vs 225	NS			18 of 238	Unclear, 10 years display
Wu et al 2019	Whole				**CD274**: Dako (1:50)	I – IV	Manual	>1% positive	Membrane	84 vs 120	Y	MV		NA	22 months
Chen et al 2020	Whole				**CD274**: Abcam	I – III	Manual	Immunoreactivity score >3	Any	94 vs 31	Y	UV[1]	-ve	NA	Unclear, 5 years display

PDCD1/CD274 combined tumour tissue and immune cell expression and overall survival in colorectal cancer

Study	**TMA or Whole section**	**If TMA …**			**Antibodies used**	**Cohort assessed (stage)**	**Measu-rement**	**Threshold**	**Tumour region**	**Groups (overall or high to low)**	**Significant**	**UV or MV**	**Effect**	**MMR**	**Median follow up**
		How many cores?	**Size of cores**	**Choice of core**											

Wei et al 2018	TMA	Unclear	0.6mm	Repres-entative	**CD274**: SP142, spring bioscience)	I – IV	Manual	Semiquantitative	ST	162 vs 191	Y	UV[m]	+ve	97 of 354	72 months
Bae et al 2018	TMA	1	3mm	Repres-entative	**CD274:** AnaSpec	I – IV	Manual	50% staining	Uncl	93 vs 82	Y	UV[n]	+ve	NA	88 months

[†]18 MSIH patients crossover, different antibody [a]no MV given [b]not independent of T-stage/CEA [d]not independent of CD274 on tumour cells [d]no MV performed. Worse prognosis for high PDCD1 TILs in pulmonary metastasis [e]not independent of age/sex/T-stage/N-stage/M-stage/tumour grade/VI [f]not independent of CD274 in stroma/tumour cells [g]not independent of age/stage/gender/MMR/VI [h]not independent of M-stage [j]not independent of age/gender/T-stage/N-stage/M-stage [k]not independent of T-stage/CEA [l]no MV given [m]not independent of tumour diff/T-stage/N-stage/M-stage/gender [n]not independent of T-stage/perinodal extension

Table A3.7. Colorectal cancer-specific survival and PDCD1/CD274 expression

PDCD1/CD274 tumour tissue expression and cancer-specific survival in colorectal cancer															
Study	**TMA or Whole section**	**If TMA …**			**Antibodies used**	**Cohort assessed (stage)**	**Measu-rement**	**Thres hold**	**Tumour region**	**Groups (overall or high to low)**	**Signifi cant**	**UV or MV**	**Effec t**	**M MR**	**Median follow up**
		How many cores?	**Size of cores**	**Choic e of core**											
Hamada et al 2017	TMA	2-4	0.6mm	Unclea r	**CD274**: CD274, eBioscienc e	I – IV	Manual	Semiq uantita tive	Cytoplasm + membrane	384 vs 233	NS			108 of 601	11.5 years

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