An investigation into the role of faecal calprotectin assessment in the diagnosis and management of colorectal neoplasia

By

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Abstract

Colorectal cancer is the 2nd most common cause of cancer death in the United Kingdom and globally. Although staging and prognosis is based on the tumour, nodes, metastases assessment the role of both local colonic inflammation and systemic inflammation is now recognised as an important component of determining cancer progression and survival. Faecal calprotectin (FC), a measure of colonic inflammation, represents another assessment of inflammation. Whether colonic inflammation measured by FC has a role in development or progression of colorectal cancer (CRC) is not known. Furthermore, it is unclear whether existing measures of local and systemic inflammation relate to colonic inflammation measured by FC.

The work presented in this thesis investigates the correlation with faecal calprotectin and colorectal neoplasia, systemic inflammation and the tumour microenvironment. I hypothesised that faecal calprotectin levels are associated with presence of colorectal neoplasia and a diagnosis of colorectal cancer, correlating with advancing disease stage and the presence of local peritumoural inflammation.

In chapter 3, a systematic review of the published literature demonstrated that the role of FC in the diagnosis of CRC has not been defined. There is a lack of evidence supporting an association between FC and adenoma/advanced adenomas. However my review confirmed an association between FC and CRC where median FC was higher in CRC, in comparison to healthy subjects in fifteen of the sixteen studies and a 5-fold increased likelihood than controls to have an elevated FC (OR 5.19, 95% CI 3.12-8.62, P<0.001 with a heterogeneity (I^2=27%)).

In chapter 4, I studied the role of FC in a large, well defined cohort of faecal occult blood test (FOBT) positive patients as part of a screened population. In this study, FC was strongly associated with CRC (sensitivity 92.8% for CRC, at 50µg/g) but lacked specificity. FC also failed to show sufficient sensitivity and specificity for the detection of non-cancer neoplasia. In chapter 5 within this screening cohort, I evaluated the relationships between FC and systemic markers of inflammation, but found no evidence of a strong link between a systemic inflammatory response (SIR) and presence of CRC and no significant relationship between FC and SIR.

In chapter 6, a larger cohort of CRC patients in whom FC measurement was performed, advanced disease stage had a possible non-significant association with higher levels of FC, with T4 tumours having the highest median FC (321µg/g), with 67% having a FC ≥200µg/g. 29% of those with T1 tumours had a FC ≥200µg/g. Patients with nodal or metastatic disease had higher median FC, compared to those without. Patients with peritoneal involvement had significantly higher median FC, compared to those without, median FC (405µg/g vs 164µg/g), p <0.05. 89% of patients with peritoneal involvement had FC ≥200µg/g, compared to 44% in those without peritoneal

involvement ($p<0.05$). Poorly differentiated tumours had a higher median FC (281.5µg/g) than well/moderate differentiated tumours (169µg/g), but not significantly. Patients with larger tumours had higher FC levels, tumours ≥3.5cm had a higher median FC 251.5µg/g, and 67% had a FC ≥200µg/g, in comparison to those with a tumour <3.5cm (median 164 µg/g, and 48% FC ≥200µg/g). To summarise larger, more advanced tumours were more likely to have higher levels of FC.

In chapter 7, in the context of a pilot study, I assessed whether there was an association between FC and markers of the local inflammatory response. I found that both Klintrup-Mäkinen (KM) and tumour stroma percentage (TSP) have higher FC levels in high grade KM and TSP, in comparison to low grade. As a preliminary study these results, suggest that there may be an association between FC, KM and TSP, which warrants further study.

In summary, this thesis has confirmed an association between FC and CRC and potentially in larger, more advanced tumours in CRC. There may be an association between FC and local peritumoural inflammation in the tumour microenvironment in CRC. More work is required to clarify if FC can safely be used in the prioritisation of patients requiring CRC diagnostic investigations and in the staging of CRC as a marker of more advanced disease and a marker of the local inflammatory response.

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Authors Declaration

The work presented in this thesis was undertaken during a period of research between 2015 and 2017 in the Academic Unit of Surgery, Glasgow Royal Infirmary, University of Glasgow. The work has been completed whilst working as a General Surgery Specialty Trainee in the West of Scotland.

I declare that the work presented in this thesis was undertaken by myself, except where indicated below:

- The parent study 'Investigation of the local and systemic inflammatory response and of dietary habits in those attending for investigation via the National Health Service Colorectal Cancer Screening Programme' was originated by Ms Cariss Little.

- Assistance with patient recruitment for the above study by Mr Domenic Di Rollo

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- Tumour microenvironment scoring for chapter 7 was performed by Dr Joanne Edwards, and her team including Phimmada Hatthakarnkul.

Publications and Presentations

Publications

The role of faecal calprotectin in diagnosis and staging of colorectal neoplasia: a systematic review and meta-analysis.

Ross FA, Park JH, Mansouri D, Combet E, Horgan PG, McMillan DC, Roxburgh CSD. BMC gastroenterology. 2022;22(1):176. doi:10.1186/s12876-022-02220-1.

The role of faecal calprotectin in the identification of colorectal neoplasia in patients attending for screening colonoscopy.

Ross FA, Park JH, Mansouri D, Little C, Di Rollo DG, Combet E, Van Wyk H, Horgan PG, McMillan DC, Roxburgh CSD.. Colorectal disease : the official journal of the Association of Coloproctology of Great Britain and Ireland. 2022;24(2):188-96. doi:10.1111/codi.15942.

Presentations

The relationship between local adaptive and innate immune responses, the systemic inflammatory response and survival of patients with colorectal cancer

Ross FA, Park JH, Horgan PG, McMillan DC, Roxburgh CSD. Cell Symposia: Cancer, Inflammation, and Immunity Conference. 11-13 June 2017, San Diego. Poster presentation.

The prognostic value of neutrophil myeloperoxidase within the tumour microenvironment in colorectal cancer

Ross FA, Park JH, Richards CH, Horgan PG, McMillan DC, Roxburgh CSD. Association of Coloproctology of Great Britain and Ireland 2017 Annual Meeting, Bournemouth, 3-5 July 2017. Poster presentation.

The prognostic value of neutrophil myeloperoxidase within the tumour microenvironment in colorectal cancer

Ross FA, Park JH, Richards CH, Horgan PG, McMillan DC, Roxburgh CSD. Glasgow Royal Infirmary Research Prize Afternoon, 10 February 2017. Poster presentation.

A comparison of the prognostic value of the adaptive, T-lymphocyte and generalised inflammatory infiltrate in patients with primary operable colorectal cancer

Ross FA, Roxburgh CSD, McMillan DC, Horgan PG, Park JH. Glasgow Royal Infirmary Research Prize Afternoon, 10 February 2017. Poster presentation.

A comparison of the prognostic value of the adaptive, T-lymphocyte and generalised inflammatory infiltrate in patients with primary operable colorectal cancer

Ross FA, Roxburgh CSD, McMillan DC, Horgan PG, Park JH. NCRI Cancer Conference, Liverpool, 6-9 November 2016. Poster presentation.

Dedication

To all my friends and family for their unwavering love and support during this period of research and completion of my thesis.

Special mention to my dad, who underwent successful treatment of colorectal cancer during this period, after an early diagnosis thanks to the bowel screening programme.

Definitions/Abbreviations

AA: advanced adenoma

ACE: angiotensin converting enzyme

AJCC: American Joint Committee on Cancer

APC: adenomatous polyposis coli

BRRS: Bannayan-Riley-Ruvalcaba Syndrome

BMI: body mass index

C: Controls

CD: Crohn's Disease

CEA: carcino-embryonic antigen

CIMP: CpG island methylator phenotype

CIN: chromosomal instability

COX: cycloxygenase

CRC: colorectal cancer

CRP: c-reactive protein

CS: Cowden Syndrome

CT: computed tomography

CTC: CT colonography

DC: dendritic cell

dMMR: DNA mismatch repair

Dx: diagnosis

EGFR: epidermal growth factor receptor

EIA: enzyme immunoassay

ELISA: enzyme-linked immunosorbent assay

FAP: Familial adenomatous polyposis

FBC: full blood count

FC: faecal calprotectin

FFQ: food frequency questionnaire

FH: family history

FIT: faecal immunochemical test

FOBT: faecal occult blood test

gFOBT: guaiac faecal occult blood test

GI: gastrointestinal

GMS: Glasgow microenvironment score

GP: general practitioner

H&E: haematoxylin and eosin

HCA: heterocyclic amines

HDI: Human Development Index

HNPCC: hereditary non-polyposis colon cancer

HRA: high risk adenoma

IBD: inflammatory bowel disease

IDA: iron deficiency anaemia

IGF: insulin-like growth factor

IL: interleukin

iNOS: inducible nitric oxide synthase

JPS: Juvenile polyposis syndrome

KM: Klintrup-Mäkinen

KRAS: Kirsten rat sarcoma viral oncogene homolog

LIR: local inflammatory response

MDSC: myeloid derived suppressor cell

mGPS: modified Glasgow prognostic score

MMR: mismatch repair

MRI: magnetic resonance imaging

MSI: microsatellite instability

NK: natural killer

NLR: neutrophil lymphocyte ratio

NPV: negative predictive value

NF-κB: nuclear factor kappa B

NLR: neutrophil lymphocyte ratio

NOC: N-nitroso compounds

NSAID: non-steroidal inflammatory drug

OGD: oesophageal-gastro duodenoscopy

PAH: polycyclic aromatic hydrocarbon

PAMP: pathogen associated molecular pattern

PHTS: PTEN hamartoma tumour syndrome

PI: Peterson Index

PJS: Peutz-Jeghers Syndrome

PLR: platelet lymphocyte ratio

PPV: positive predictive value

PPI: proton pump inhibitor

PRR: pattern recognition receptor

PS: Proteus Syndrome

PTEN: phosphatase and tensin homolog

qFIT: quantitative faecal immunochemical testing

ROS: reactive oxygen species

RNI: reactive nitrogen intermediates

SBSP: Scottish Bowel Screening Programme

SC: Screening

SIMD: Scottish Index of Multiple Deprivation

SIR: systemic inflammatory response

SP: Specific

SY: Symptomatic

TAM: tumour associated macrophages

T2DM: type 2 diabetes mellitus

TIL: tumour infiltrating lymphocytes

TME: tumour microenvironment

TNF: tumour necrosis factor

TNM: tumour nodes metastases

TSP: tumour stroma percentage

UC: Ulcerative Colitis

UK: United Kingdom

TME: tumour microenvironment

WEGF: vascular endothelial growth factor

WCC: white cell count

1.0 Introduction

1.1 Epidemiology of colorectal cancer

1.1.1 Worldwide

Globally colorectal cancer (CRC) is one of the most common cancers, accounting for 1 in 10 cancers diagnosed. [10] In 2020 it was the third most common cancer diagnosis, with 1.93 million cases diagnosed, and the second leading cause of cancer death with 935,000 deaths.[11, 10] CRC is more prevalent in males, with 55% of the cases diagnosed. [11] The highest rates occur in the developed world; North America, Europe and Australasia, with higher incidence in high/very high HDI (Human Development Index) countries with an incidence of 29 per 100.000 compared to 7.4 per 100,000 in low/medium HDI countries. [10]

1.1.2 United Kingdom and Scotland

In the United Kingdom (UK), CRC is the fourth most common cancer. [12] It accounts for 11% of all cancers diagnosed with 42,300 new cases diagnosed each year (2015-17). [12] Similar to global statistics, CRC is more common in males in the UK (56% cases are diagnosed in men). Over the past 15 years CRC incidence has decreased by 4%. [12] CRC is the second most common cause of cancer death, accounting for 10% of all cancer deaths, with 16,600 deaths each year, (2016-2018). [12] 5 year survival has improved from 24% in 1971/72 to 59% in 2010/11. [12]

In Scotland CRC was the fourth most common cancer in 2020, with 3,309 people diagnosed with colorectal cancer in. [13] In 2020 the number diagnosed fell by 19%, compared to 2019.[13] The largest reduction was seen in early stage disease, probably due to the Covid-19 pandemic and the temporary pause of the national cancer screening programme. [13] CRC is the second leading cause of cancer mortality, 10.8% of all cancer deaths in 2018. [14] 5 year survival in Scotland is comparable to the UK with 60% survival. [14]

1.2 Aetiology of colorectal cancer

The aetiology of colorectal cancer is complex, with cancers developing slowly due to a multitude of genetic, host and lifestyle factors.

1.2.1 Genetic

15-20% of colorectal cancers are diagnosed in patients with at least one first-degree relative with the disease. [15] However only 5% have recognised genetic syndromes predisposing them to colorectal cancer. [16]

1.2.1.1 Adenomatous polyposis syndrome

Familial adenomatous polyposis (FAP) is an autosomal dominant disease. Patients characteristically develop hundreds of adenomas in the colon and rectum. [17] It has an incidence of 1 in 5,000-10,000 people, with 100% of patients developing colorectal cancer if not identified and treated early. [18, 19] Overall less than 1% of all CRCs are caused by FAP. [18] FAP results from germline mutations of the tumour suppressor adenomatous polyposis coli (APC) gene, on the long arm of chromosome 5 in band q21 (5q21). [17] 75–80% of the mutations are inherited, while 15–20% result from de novo mutations.[20] Prophylactic cancer-preventive colorectal surgery is advocated for patients due to the risk of CRC development, in their teens/20s. [17] Prophylactic surgery, whilst polyps are at a pre-malignant stage, reduces the morbidity and mortality. [19] However there is still mortality from extra-colonic manifestations of FAP including desmoid tumours, and gastric and duodenal polyps. [19, 18] Gardner's syndrome is a clinical variant of FAP where the extra-colonic features are prominent, but is not genetically distinct from FAP. [17, 18]

1.2.1.2 Non polyposis syndrome

Hereditary non-polyposis colon cancer (HNPCC, or Lynch syndrome) has a frequency of 1:370 to 1:2,000. [21] It is associated with a 50-80% lifetime risk of developing CRC, although different risk is emerging for different involved genes. [18, 21] 3% of all colorectal cancers are caused by HNPCC. [15] HNPCC is an autosomal dominant condition associated with germline mutations in multiple DNA mismatch repair (MMR) genes responsible for repairing DNA replication errors, arising through the microsatellite instability (MSI) pathway. [15, 22] Patients develop CRC at a younger age than patients with sporadic CRC (typically in their 4^{th} or 5^{th} decade) and are also at risk of developing extra colonic pathology including cancers of the endometrium and ovaries. [18]

1.2.1.3 Hamartomatous polyposis syndromes

The hamartomatous polyposis syndromes are rare genetic syndromes characterised by the development of hamartomatous polyps in the gastrointestinal tract. They subsequently cause the development of colorectal cancers (<1% of all CRCs) including Peutz-Jeghers syndrome (PJS),

Juvenile polyposis syndrome (JPS), phosphatase and tensin homolog (PTEN) hamartoma tumour syndrome (PHTS) as well as hereditary mixed polyposis syndrome. [23, 24]

PJS is an autosomal dominant condition, with germline mutations in tumour suppressor STK11 gene. It has an incidence of between 1:8,300 and 1:200,000 and is characterised by mucocutanous melanosis and polyposis of the GI-tract. Patients with PJS have an increased risk of both colonic and extra colonic cancer (including stomach, small bowel, pancreas, breast and gynaecological) with the overall cumulative risk for cancer more than 90%. [23, 24] With a relative risk of 84 for development of CRC. [24]

JPS is an autosomal dominant condition with mutations in the transforming growth factor (TGF-beta) pathway including BMPR1A, SMAD4 and ENG. [23, 24] The incidence is approximately between 1:100,000 to1:160,000 and is characterized by the occurrence of multiple juvenile polyps in the GI-tract, with majority in colon and rectum (less than 20% occur in the stomach and small intestine). [23, 24] The polyps are named based on their histological appearance (gland dilatation, inflammatory cell infiltrate and absence of smooth-muscle proliferation) unrelated to age. [24] Colorectal cancer is the most common malignancy that develops in JPS, with a lifetime risk of 17–22% by age 35 and approximately 68% by age 60. [18, 24]

PTEN hamartoma Tumour syndrome (PHTS) includes Cowden syndrome (CS), Bannayan-Riley-Ruvalcaba syndrome (BRRS), PTEN-related Proteus syndrome (PS), and Proteus-like syndrome. This is the umbrella term for a group of disorders all caused by germline mutations of the tumour suppressor gene. [24] The increased risk of malignancy (particularly breast, endometrial and thyroid) is well described in CS. [23, 24] However, among the different PHTS disorders, colorectal cancer risk is unclear with a possible lifetime risk of up to 9% reported. [24, 18]

1.2.1.4 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD), incorporating ulcerative colitis (UC) and Crohn's disease (CD) is a risk factor for CRC. In UC, CRC prevalence has been reported at 3.7%. [25] This increases with duration of disease with risk of CRC 8% at 20 years, and 18% at 30 years. [25] In CD, the risk of CRC is less defined and may vary with disease distribution. [26] Prevalence has been reported as a RR of 0.80 to more than 20. [26] Overall CRC is a major cause of death in IBD accounting for 10-15% of mortality. [27] IBD itself develops as a multifactorial result of genetic, immunological and environmental factors. CRC develops in IBD as a consequence of chronic inflammation, in which non-polypoid dysplasia occurs as a result of p53 mutations, hypermethylation of the MLH1 gene, and MSI. [27, 28] Production of inflammatory mediators such as tumour necrosis factor (TNF) promotes activation of nuclear factor kappa B (NF-κB) signalling upregulating anti-apoptotic signals. [27, 28]

1.2.2 Host

With only 20% of CRCs being genetic in origin, the majority (80%) are sporadic. Therefore it is evident that both the host and the environment also play an important role in carcinogenesis of CRC. CRC can be considered a marker of socio-economic development. [10] CRC rates increase as a reflection of higher socio-economic status and ensuing lifestyle changes. [10]

1.2.2.1 Age

The majority of CRCs are diagnosed in patients over 50 years of age. [29] In the UK age specific incidence of CRC rises from around age 50, with the highest rate at age 85-89 years. [12] The Scottish Bowel Cancer Screening Programme commences at age 50, correlating with the rise in CRC rates at this age. [30] With increasing age comes increased time for exposure to the multifactorial genetic, host and environmental risks for developing cancer. Increasing age also causes changes in molecular pathways accelerating the initiation of mutations in stem and progenitor cells resulting in failure of cancer suppression. [31] Increasing age is also associated with chromosomal telomeres degradation and epigenetic changes resulting in chromosomal instability and predisposition to mutations. [32]

1.2.2.2 Obesity / metabolic syndrome

High body weight is an independent risk factor for CRC, in obesity a relative risk of 1.24 for men, and is causative aetiology in 11% of CRC cases. [33, 34] Metabolic syndrome is a set of risk factors combining visceral obesity with dyslipidemia, elevated fasting plasma glucose and hypertension is associated with increased CRC risk. [35, 36] This is multifactorial and thought to be due to alterations in adipokine secretion and cell signalling pathways, tumour promoting effects of insulin-like growth factor-1 and secretion of proinflammatory cytokines by adipose tissue creating an inflammatory and pro-tumour environment. [35-37]

1.2.2.3 Physical inactivity

Physical inactivity is associated with a higher risk of developing colorectal cancer and is thought to be a causative aetiology in 10% of patients and increases an individual's risk of developing CRC by 50%. [38, 29] While physical activity is associated with a reduced risk of CRC, by approximately 27%. [39] It has been shown that while physical activity and its benefits can be linked to other CRC risk factors including weight, cardiovascular disease and diabetes, it is also independently beneficial. [40]

1.2.2.4 Cardiovascular disease

Patients with cardiovascular disease have an increased risk of developing CRC. [41] Both diseases share pathophysiology and risk factors, which may explain this association. [42, 43] Chronic

inflammation has been linked to both atherosclerosis and carcinogenesis. Shared common risk factors include obesity, metabolic syndrome, physical inactivity, smoking and diet.

1.2.2.5 Diabetes

Diabetes is associated with an increased risk of CRC. [44-46] Similar to cardiovascular disease, diabetes and CRC share risk factors for development of disease and the associated risk can reflect confounding. However Larsson et al showed that the association remained when controlling for body mass index (BMI) and physical activity. [46] Diabetes (particularly type 2) is associated with insulin resistance, subsequent hyperinsulinaemia, and resultant circulating insulin-like growth factors (IGFs). IGFs are peptides that regulate cell proliferation, differentiation and apoptosis. [47] Therefore increased circulating IGF in diabetes results in inhibition of apoptosis and promotion of pro-inflammatory cytokines including TNF-α and interleukin-6 (IL-6), resulting in colorectal carcinogenesis. [48, 47]

1.2.2.6 Systemic Inflammatory Response

Inflammation is known as a hallmark of cancer promoting tumour proliferation and dissemination. The presence of an elevated systemic inflammatory response (SIR) is associated with increased risk of CRC.

Inflammation results in tissue damage causing increased cytokine production (TNF-α and ILs). This is turn activates the NF-κB pathway, which regulates multiple factors involved in carcinogenesis including inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2 resulting in angiogenesis and immunosuppression. Inflammation also results in production of reactive oxygen species (ROS), with overproduction resulting in DNA and tissue injury.

SIR is associated with an increased burden of disease, and in patients who have undergone potentially curative resection for colorectal cancer, the presence of a SIR predicts a poor outcome. Presence of an elevated systemic inflammatory response has been consistently associated with reduced survival, independent of stage, across a number of cancers including colorectal cancer. [49-54] Inflammation is discussed in more detail in section 1.7.

1.2.3 Environmental

Diet

There are multiple elements of diet that can contribute to the risk of CRC development.

1.2.3.1 Animal source foods

Red meat and particularly processed meat have been shown in meta-analyses to be associated with CRC. [55-57] The reasoning for this is likely multifactorial and resultant from carcinogens present in meat including heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs) produced when meat is cooked at high temperatures. In this process, haem iron acts as a catalyst by promoting the stimulation of N-nitroso compounds (NOCs) and resultant mutations in Kirsten rat sarcoma viral oncogene homolog (KRAS) and APC. [55, 58]

1.2.3.2 Fibre

Wholegrains and consequently fibre are protective against CRC. [55, 59, 57, 60] Multifactorial elements occur to reduce the risk of CRC, including the multiple compounds found in fibre; vitamin E, selenium, copper, zinc and phenolic which all may have anti-carcinogenic properties. Fibre is fermented in the bowel, forming short chain fatty acids which may have anti-proliferative effects. Fibre also decreases colonic transit time, reducing exposure to carcinogenic compounds, as well as reducing insulin resistance: is a risk factor for CRC. [55, 59]

1.2.3.3 Fruit and Vegetables

Increased consumption of fruit and vegetables is associated with reduced risk of CRC. [61, 55] Fruit and vegetables provide the host with micronutrients and phytochemicals – these have demonstrated anti-cancer effects in studies. They can also contain dietary fibre, which as discussed above is a protective factor for CRC. Polyphenols are phytochemicals in plants which are found in many foods including many components of the Mediterranean diet such as olive oil, fruits, vegetables.[62] Polyphenols have a protective effect against CRC. [63, 62] This is thought to be due to the chemoprotective effects, by interfering with progression of carcinogenesis by downregulation of COX-2, BCL-2 and EGFR expression. [63]

1.2.3.4 Alcohol

Alcohol is associated with increased risk of CRC, in a dose dependent manner. [57, 64, 65] Multiple mechanisms are thought to be at play including metabolism of alcohol to acetaldehyde, the carcinogenic effect of nitrosamines, and increased degradation of folate. [64]

1.2.3.5 Smoking

Smoking is a risk factor for cancer and more recently confirmed to be a risk factor for CRC, in a dose dependent manner. [66, 67]

1.2.3.6 Calcium and vitamin D supplements

The link between calcium, vitamin D and CRC remains unproven, but the evidence available suggests that calcium and vitamin D supplementation is protective for CRC. [68, 55] One meta-analysis showed a RR 0.70 (95% CI 0.58 to 0.84), p=0.0002 for vitamin D (25OHD) in colorectal cancer. [68] A meta-analysis showed an inverse association for CRC for high vs low levels of calcium intake (RR 0.76 (95%CI 0.65-08.9)). [55, 69] The mechanisms proposed for these protective factors include prevention of K-ras mutations, promotion of cell differentiation and improvement of the immune response. [70-73]

1.2.3.7 Aspirin and non-steroidal anti-inflammatory drugs

Aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to lower the risk of colorectal cancer. [74] This was first reported in the 1980's and there is now strong evidence for the role of aspirin in CRC prevention. [75-77] In 2010 Rothwell et al, published results of multi RCTs showing that low dose aspirin for 5+ years reduced overall colorectal cancer incidence and mortality, with greatest benefit seen in right sided cancers when risk reduced by 70%. [78] Aspirin and NSAIDs suppress COX-activity and thus prostaglandin synthesis. Abnormal COX-2 and prostaglandin expression has pro-tumour effects; increase in tumour cell proliferation, decreased apoptosis and increased angiogenesis. [79] NSAIDs are also thought to cause non COX mediated interactions with signal pathways, including Wnt/β-catenin, NF-κB and phosphatidylinositide 3-kinase/AKT. [79] The anti-inflammatory action may also have a direct effect on the microenvironment and inflammatory response, by increasing tumour infiltration of activated T-lymphocytes and a decrease in immunosuppressive regulatory T-lymphocytes. [79, 80]

1.3 Carcinogenesis of colorectal cancer

CRC arises from a combination of different molecular and genetic pathways including microsatellite instability (MSI), chromosomal instability (CIN) and methylation pathway (CIMP), as well as the classic adenoma-carcinoma sequence. This is summarised in Figure 1.1.

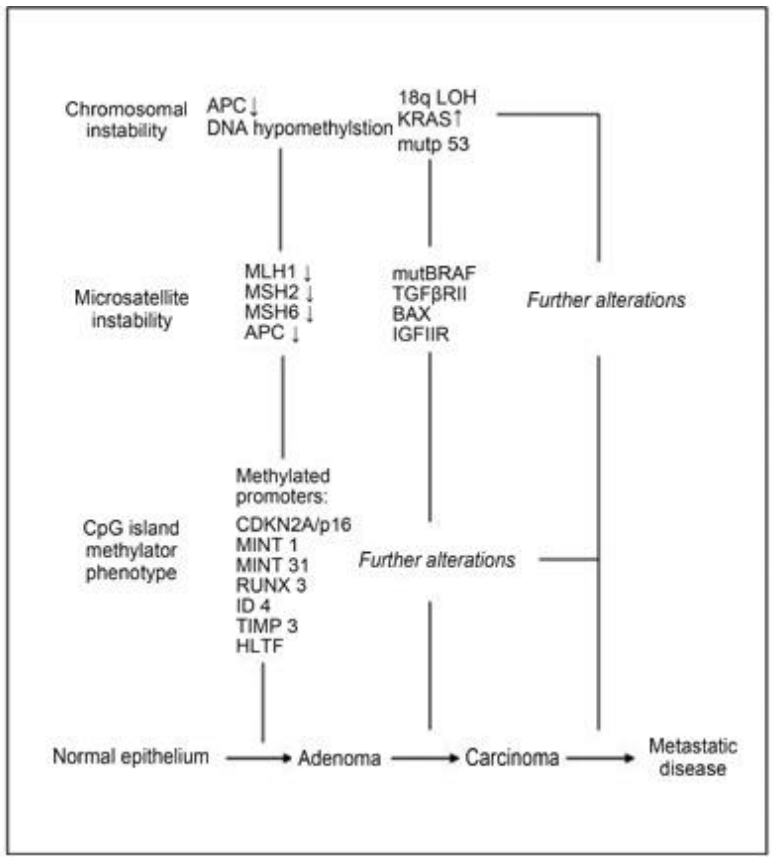

Figure 1.1. Summary of CRC carcinogenesis.
Figure from Tariq et al 2016, used with permission from Creative Commons Attribution License 4.0 [8]

1.3.1 Adenoma-carcinoma sequence

Over 90% of CRCs arise from dysplastic adenomatous polyps, and transform into cancers via the adenoma-carcinoma sequence of pathogenesis. [81, 82] This describes the development of adenomas and their progression to invasive malignancy, was originally described in 1988 by Vogelstein et al. [83] Figure 1.2 shows the pathway consisting of a number of mutations occurring in the transition from adenoma to cancer. [84]

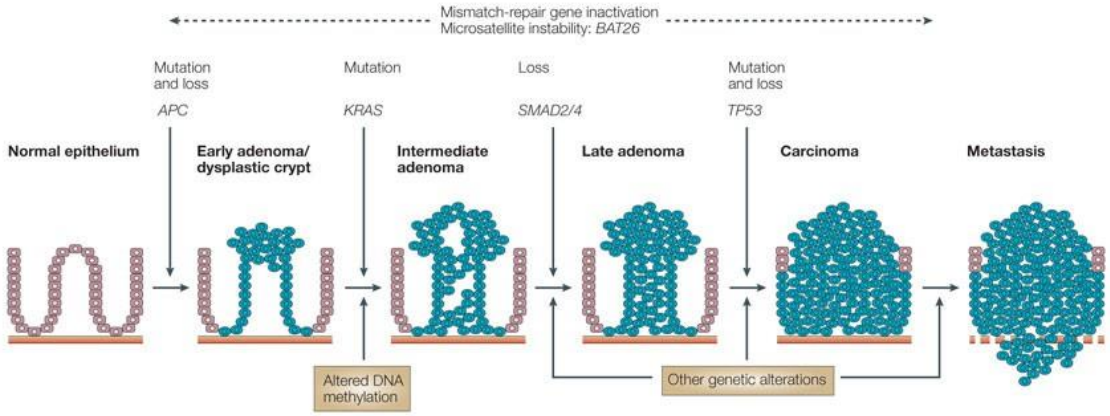

Figure 1.2. Adenoma-carcinoma sequence.
Figure from Davies et al 2005, used with permission. [3]

1.3.2 Chromosomal instability

The CIN pathway begins with mutations in the APC, followed by the mutational activation of oncogene K-ras and the loss of tumour suppressor gene p53. [8] However these steps may not always occur, nor always occur in the same order.

Deletion of the APC gene is found in 70-80% of polyps. [85, 86]. This occurs though mutation or loss of chromosome 5q. [86] This leads to truncation of the APC protein which results in higher levels of ß-catenin protein accumulating, leading to deregulation of the Wnt-APC-ß-catenin signalling pathway. [81] APC mutations are seen in 80% of both adenomas and CRCs. [84]

K-ras is also discussed in section 1.6.3.2. K-ras (Kirsten rat sarcoma viral oncogene homolog) is a member of the RAS family. Mutations have been found in approximately 40% of colorectal adenomas, and are thought to occur in the early stages of adenoma development. Mutations in the K-ras oncogene (codons 12 and 13) lead to activated K-ras protein, uncontrolled cell proliferation and progressive dysplastic changes to an adenoma. [87-91, 81, 8, 84]

The SMAD4 protein is involved in signal transduction in the beta-transforming growth factor growth inhibitory signalling pathway. In carcinogenesis the SMAD4 gene is inactivated by loss of a part of chromosome 18q (where it resides) or by gene mutation. This results in loss of inhibitory signals and therefore allows increased tumour cell growth. Chromosome 18q, is lost in approximately 10-30% of early adenomas increases up to approximately 60% in late adenomas. [81, 84]

This is traditionally followed by loss of the p53 tumour suppressor gene, located on the short arm of chromosome 17. The p53 gene encodes the p53 protein, which responds to DNA damage by upregulation of the CDK inhibitor p21, ultimately causing cell cycle arrest and an opportunity to

repair the DNA damage. p53 mutation and loss is seen in less than 26% of adenomas, but in approximately 50% of invasive foci within adenomatous polyps and up to 75% of CRCs. [81, 91, 8, 84]

1.3.3 Microsatellite instability

MSI is also discussed in section 1.6.3.3. Microsatellite instability (MSI) is change in the number of repeats or length (due to nucleotide insertions or deletions) of normal segments of DNA with repeat sequences of nucleotides of set length. MSI is caused by defective DNA mismatch repair (dMMR) genes. This defective dMMR is caused by germline mutational inactivation of genes encoding MMR proteins. A five-marker MSI panel has been validated, which includes five microsatellites; two mononucleotide markers, BAT25 and BAT26, and three dinucleotide markers, D5S346, D2S123, and D17S250. Tumours are graded high frequency when MSI >30% of the panel is mutated. [89, 87, 92-95, 8] MMR testing is measured via immunohistochemistry to detect expression of MMR proteins MLH1, MSH2, MSH6 and PMS2. [96]

1.3.4 CpG island methylation pathway

The CpG island methylator phenotype (CIMP) pathway is characterized by DNA methylation of tumour suppressor genes, silencing gene transcription and essentially impeding tumour suppressor genes. In CRC, hypermethylation gives rise to the CIMP. If a tumour has a significant proportion of genes with hypermethylated CpG islands, the tumour is designated as CIMP-positive. CIMP CRC is thought to arise from serrated hyperplastic colonic polyps. This is associated with BRAF mutation and MSI. [8, 81]

1.4 Clinical presentation

Colorectal cancer is diagnosed via multiple pathways. In symptomatic patients this occurs via elective outpatient or emergency pathways. However a number of CRCs are diagnosed when the patient is asymptomatic via the bowel screening programme.

1.4.1 Symptomatic

The majority of CRCs diagnosed in the UK are in patients who are symptomatic (90%) and present electively (67%), but 23% still present as an emergency presentation. [97] This number has reduced, from 31%, in a 2004 pre bowel screening era study of more than 3000 CRC patients. [98] It has been reported that patients presenting as an emergency are more likely to be aged over 75 years and female. [98] One study reported that approximately 60% of emergency CRC admissions and diagnoses had described at least one symptom of CRC to their general practitioner (GP) in the month prior. [99] The symptoms they reported included abdominal pain with weight loss or diarrhoea. [99] As an emergency these patients present with bleeding, perforation and obstruction. [100] Obstruction is also the most common reason for emergency surgery in CRC, occurring in 77%. [101] Perforation is the second most common reason for emergency surgery in CRC occurring in 2.6% to 12% with a high mortality up to 50%. [100] Emergency presentation is associated with higher stage of disease and subsequently both a higher 30 day post-operative mortality and a lower 5 year survival.[98, 102] The elective clinical presentation is typically with a combination of symptoms, and often dictated by the tumour location. [103] The three primary symptoms are rectal bleeding, change in bowel habit and abdominal pain. [103] Distal tumours present more commonly with rectal bleeding and change in bowel habit, while patients with more proximal tumours may present with symptoms of weight loss and iron deficiency anaemia [104]. In Scotland the national SIGN guidelines recommend that patients are referred with suspicion of CRC if they are $\geq$ 40 years old with new onset, persistent or recurrent rectal bleeding and all patients with unexplained iron deficiency anaemia should be referred for endoscopic investigation. [105]

1.4.2 Bowel screening programme

In the UK, only 10% of CRCs are diagnosed via bowel screening. [97] In June 2007 the Scottish Bowel Screening Programme commenced and by December 2009, Scotland became the first country in the world to have a fully rolled out a bowel screening programme. [106] Patients between the age of 50 and 74 and registered with a GP, are invited to participate. Patients aged over 74 years can opt in to participate. Every 2 years the patients are sent a faecal testing kit. [107] Since November 2017 this is a faecal immunochemical test (FIT) kit, guaiac faecal occult blood tests (gFOBT) were used prior to this.[107] The patients complete the kit at home, which involves collecting one stool sample and then returning the kit to the central screening centre in Dundee for analysis. [107] The cut-off levels for a positive result are 80 µg Hb/g faeces (initial threshold of 120µg in England). [108, 109] Patients with a positive FIT result are referred to their local hospital

for assessment by a bowel screening endoscopy nurse and then, if suitable, are invited for a colonoscopy for further investigation.

In the Scottish bowel cancer screening programme, the transition from gFOBT to FIT, reported that overall positivity increased with more colorectal neoplasia's detected with the use of FIT. There was a lower positive predictive value (PPV) for CRC but a higher PPV for adenoma, in comparison to gFOBT. [109] A 2017 meta-analysis showed an average sensitivity of FIT for CRC was 93% with a specificity of 91%, with the optimal FIT cut-off values determined to be between 15 and 25 μg/g faeces. [110]

In Scotland, of the patients invited to participate in bowel screening, 63.9% uptake was reported following introduction of FIT. 2.6% of these patients had a positive FIT, and 77.3% (of that 2.6%) went on to have a screening colonoscopy. Of the patients who had a positive FIT test 43.5% had an adenoma, and 5.2% had CRC. This is less than half of the predicted figure of 12% that was determined in a pre-bowel cancer screening pilot study having CRC. [111, 112]

Advantages of FIT over gFOBT include enhanced uptake, specificity for human haemoglobin, automated reading and quantification of haemoglobin in faeces. [109] There is also good evidence that FIT results in higher neoplasia detection rates. [109] The high specificity and sensitivity of FIT allows patients with positive results to be investigated as a matter of priority. [110] The target

uptake of bowel screening in Scotland is 60%. Uptake is lower in the most deprived areas (46.5% vs 68.9%), and in men (56.8% vs 62.2%). [111]

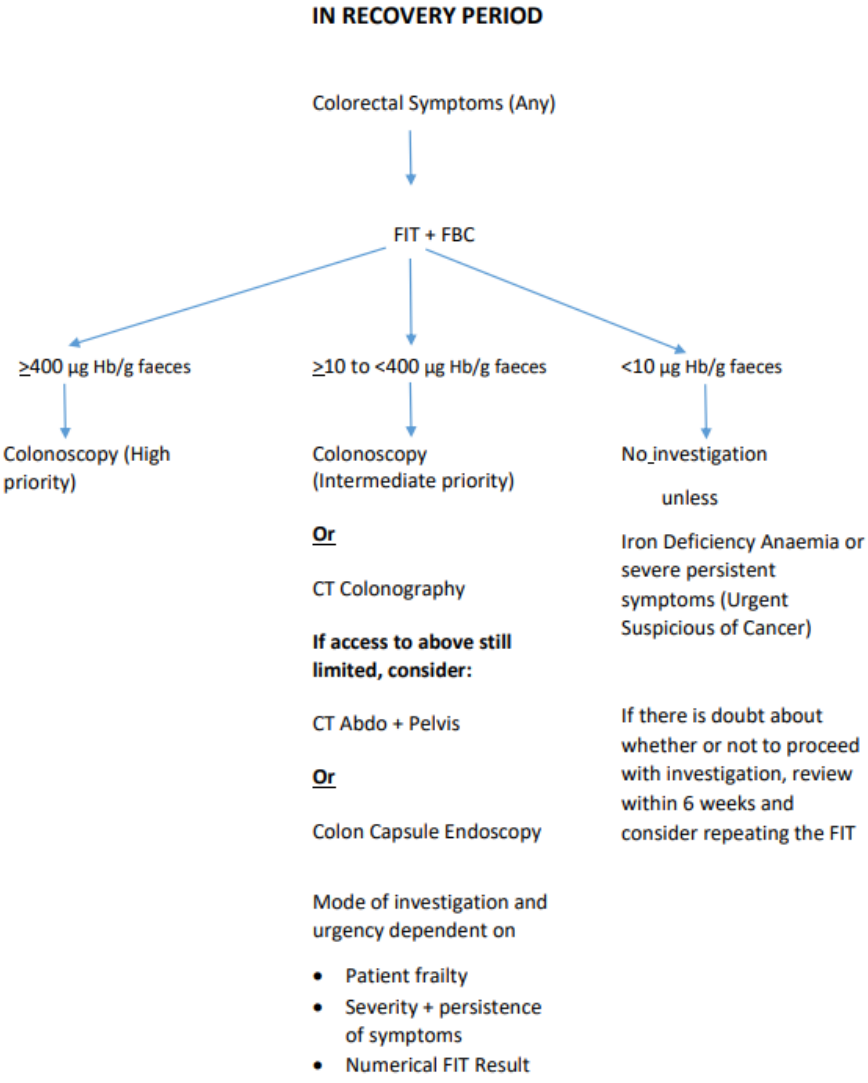

Figure 1.3. Use of FIT to investigate colorectal symptoms.
Figure from Scottish Government 2020, used with permission - Contains public sector information licensed under the Open Government Licence v3.0. [6]

Quantitative faecal immunochemical testing (qFIT) has been introduced into clinical practice in Scotland within the last few years, as part of the investigation of symptomatic patients. Figure 1.3 shows the flowchart of how the FIT result is managed. A positive result being 10 µg Hb/g faeces and above. During the Covid-19 pandemic the Scottish Government released updated guidelines on the use of qFIT (in addition to clinical acumen), to prioritise the investigations of patients with highest risk of colorectal cancer. [6] With evidence showing that an f-Hb of >400 µg Hb/g faeces has a positive predictive value (PPV), for CRC, of 22.8%, and an f-Hb of <10 µg Hb/g faeces has a PPV for CRC of 0.7%. This led to guidance to prioritise patients with a qFIT >400 µg Hb/g. [6]

Cancer screening, and in this case bowel cancer screening, is set up with the aim of detecting cancer (or pre-cancerous change i.e. adenomas) in patient without symptoms. In doing so the aim is

to remove adenomas and prevent progression to cancer, and to reduce cancer mortality by earlier treatment of cancers detected at an earlier stage. The principles of screening initiate from Wilner and Jugner 1968, who listed ten key elements of population screening; condition should be an important health problem, there should be accepted treatment for the condition, facilities for diagnosis and treatment should be available, recognisable early symptomatic phase, there is a suitable test, test need to be acceptable to the population, condition needs to be well understood, agreed policy on whom to treat, cost should be economically balanced, should be a continuous process. [113]

Colorectal cancer is a good target for population screening, as it fulfils the above elements. As discussed previously, CRCs develop from benign precursor lesions (adenomas), which progress slowly over a period of approximately 10 years to develop into cancer, allowing them to be targeted at endoscopy for early detection and removal. [114]

The ideal test for screening, would identify all of the patients with the condition from those without it. However this is not feasible and all tests will have false positives and false negatives. Therefore in real world terms, the ideal test is one with a low rate of both false positives and negatives, but also financially viable and accepted by the patients. Screening tests for CRC and bowel screening currently rely on the faecal testing discussed above (gFOBT/FIT). Other potential modalities include colonoscopy and sigmoidoscopy. Meta-analyses show that FOBT screening reduces CRC mortality by 16% compared with 30% for flexible sigmoidoscopy screening. [114] Faecal testing is cheap and non-invasive, but there are larger numbers of false-positive tests. [114] Flexible sigmoidoscopy is more expensive, and invasive, but is effective for once-only screening. [114] Overall, endoscopic tests may be more specific and sensitive than faecal testing, but at the expense of being more invasive, and costly.

1.5 Diagnosis and management

1.5.1 Investigations

A diagnosis of CRC is typically made histologically from biopsy samples taken during endoscopy. However the exact investigations (and the timing and order) performed to get to the diagnosis is dependent upon many factors including, symptoms, patient fitness and mode of presentation.

1.5.1.1 Laboratory tests

As discussed in 1.4.1, in Scotland, FIT is primarily used as a screening test, however in the past year it has become used in clinical practice to prioritise the investigations of patients with highest risk of colorectal cancer for further investigations. GPs will ask patients who present to them with symptoms of CRC, to provide a faecal sample for qFIT. This guides vetting of patients when referred to hospital, and what patients can be sent directly for endoscopic investigation. [6] No other faecal tests are used in routine clinical practice as part of the diagnosis of CRC.

Carcino-embryonic antigen (CEA) has not been shown to be of value in diagnosing early CRC. However high preoperative concentrations of CEA may correlate with adverse prognosis and serial use of CEA is used to follow up patient's post-surgical resection. [115, 116] CEA is discussed in more detail in section 1.6.3.1.

The recommendations are for all patients with symptoms suspicious of CRC should have a full blood count (FBC) performed, and if anaemic further testing is required to determine the presence of iron deficiency. All patients with unexplained iron deficiency anaemia should be referred for endoscopic investigation of upper and lower gastrointestinal tract. [105]

1.5.1.2 Endoscopic

Colonoscopy is the recommended diagnostic investigation, because it is both a very sensitive method of diagnosing CRC and it also enables biopsy for a histological diagnosis. It also allows therapeutic management to be performed e.g. removal of polyps. Synchronous cancers are present in about 2–4% of patients with CRC, and it is therefore recommended that patients undergo examination of the whole colon. If this cannot be done pre-operatively (e.g. obstructing tumour) then it should be carried out in the 6 months post-operatively. Potential complications of colonoscopy include bleeding, bowel perforation and death, with a perforation rate of 1:769. [105, 117, 118]

In Scotland, colon capsule endoscopy, is being introduced as an alternative to colonoscopy. This is being termed 'The ScotCap Test' during which the patients will swallow a capsule containing two cameras to visualise the colon. The negatives are that no histological diagnosis is obtained, and if any abnormalities or uncertainties are seen then the patient will ultimately require to undergo a

colonoscopy. A recent systematic review showed that the accuracy of capsule colonoscopy was comparable to colonoscopy and superior to CT colonography, without any complications. However completion rates varied from 57 % to 92 %. [119, 120]

1.5.1.3 Imaging

Computed tomography (CT) can be used in multiple ways in CRC. Firstly a CT colonography (CTC) can be used to diagnose CRC. This has superseded double contrast barium enema in the radiological investigations of CRC. CTC has been shown to be highly sensitive for CRC, 96.1% sensitivity in a meta-analysis. CTC can be useful in frail or elderly patients who may not tolerate colonoscopy. Similar to capsule colonoscopy it does not allow histological diagnosis and if abnormalities are seen then the patient will require a colonoscopy. CT is also used to image the chest, abdomen, and pelvis as part of the full staging for local extent and distal metastases in CRC, and may also be used as the primary investigation in patients presenting as an emergency. [105, 121, 122]

Magnetic resonance imaging (MRI) is used as part of the local staging in rectal cancer. MRI of the rectum is superior to CT for this purpose, providing accurate information in tumour and nodal staging, extramural venous invasion and circumferential resection margin status. [105, 123]

1.5.2 Management

For patients with primary resectable CRC, the components of clinical management can be broken down into the neoadjuvant, surgical and adjuvant settings.

1.5.2.1 Neoadjuvant therapy

Neoadjuvant (pre-operative) radiotherapy or chemo-radiotherapy is a management option in rectal cancers, where there is concern about an involved circumferential margin if the patient was to proceed directly to surgery. Short course neoadjuvant radiotherapy reduces local recurrence, compared to adjuvant (post-operative) radiotherapy. Chemotherapy, in addition to radiotherapy, improves the complete pathological response rate and local control compared to radiotherapy alone in resectable stage II and III rectal cancer. There is no additional benefit from neoadjuvant chemo-radiotherapy in patients with stage I disease. [117, 105, 124] The use of neoadjuvant chemotherapy in colon cancer remains uncertain. Initial results from the FOXTROT trial have shown that neoadjuvant chemotherapy in colon cancer is safe, with evidence of histological down staging and reduced rate of incomplete resections (final results of this trial are yet to be published). With more evidence this may become more common practice if more evidence confirms the benefits, without risking progression of disease. [125, 126]

1.5.2.2 Surgery

Resection of the tumour is the foundation of curative treatment of colorectal cancer. The operation is performed with the aim to resect the tumour (with clear margins) and it's draining regional lymph nodes. This operation can be performed via open, laparoscopic or robotic assistance. The extent of surgery is determined by the location of the tumour and the supplying blood vessels. The standard surgical procedure for the treatment of rectal cancer is total mesorectal excision (removal of the rectum and mesorectum) because this area contains majority of the involved lymph nodes and tumour deposits, and therefore reduces the risk of local recurrence and improves survival. [117, 105, 127]

1.5.2.3 Adjuvant therapy

Adjuvant (or post-operative) treatment is currently recommended for stage III CRC, with evidence showing that adjuvant chemotherapy improves the survival of these patients. Use of adjuvant therapy in stage II CRC is based on clinical judgement taking into consideration the patients age and co-morbidities as well as their stage of disease particularly the presence of high risk prognostic factors which include T stage, presence of vascular invasion, peritoneal involvement, tumour differentiation, tumour perforation, tumour budding and margin status. [105]

1.6 Determining prognosis

Five year survival from CRC in Scotland is 60%. [8] This differs between stages of disease at diagnosis, with 91% five year survival in stage 1, compared to 10% five year survival in stage IV. [12] Even in patients who undergo planned curative resection, disease can recur in the following years, with a recurrence rate of 5% in stage I, 10-20% in stage II and 30–45% for stage III disease. [128] It is understood that a multitude of factors play into recurrence risk and survival in CRC. These include tumour factors characterised using pathology, molecular markers and the local microenvironment, as well as host factors which include age and co-morbidity.

1.6.1 Tumour staging

There are multiple systems for staging CRC. These combine staging of the tumour itself, combined with evidence of spread of disease.

1.6.1.1 Dukes' staging

This method of staging was first described by Dukes in 1932 and modified to include distant spread in 1958. [129, 2] This classification, displayed in Figure 1.4, was based upon local and lymphatic spread of disease, with the belief that lymph node metastases did not occur when tumour growth was limited to the bowel wall; A: growth limited to wall of rectum, B: extension of growth to extra rectal tissues, but no regional lymph node, C: metastases in regional lymph nodes. [2] By 1958 there was separate consideration for local spread, lymphatic spread, venous spread and tumour grade, with stage C now divided into C1: regional lymph node metastases and C2: more extensive lymph node metastases. [129, 130] A further modification was subsequently added, when stage was added to confer distant spread. [130, 131] At that time survival was reported as overall 5 year survival post resection of 48.3%, with stage dependent survival ranging from 97.7% for stage A to 13.6% for stage C2. [130, 129]

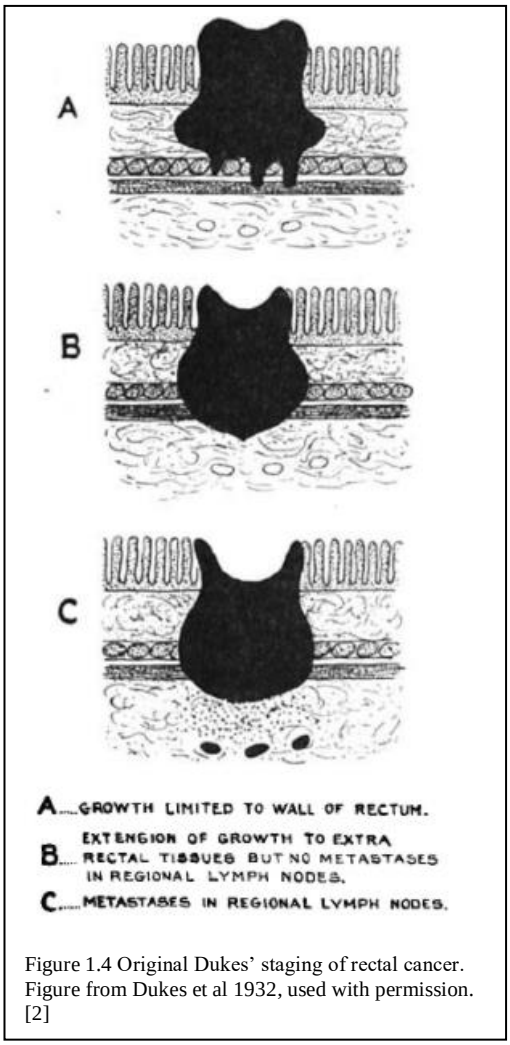

Figure 1.4 Original Dukes' staging of rectal cancer. Figure from Dukes et al 1932, used with permission. [2]

1.6.1.2 TNM staging

The tumour nodes metastases (TNM) cancer staging system is a logical description of cancer stage, and has been used to stage all localities of cancer. T describes the primary tumour site and size, N describes the regional lymph node involvement and M the presence or otherwise of distant metastatic spread. [132] While, for CRC, this staging system is similar to Dukes staging it has all but replaced it in clinical practice. The Royal College of Pathologists used TNM 5th edition routinely in the UK until 31 December 2017, and TNM 8th edition has been in use since 1 January 2018, and the Association of Coloproctology of Great Britain and Northern Ireland include it within their guidelines. [133] Table 1.1 displays five year survival in the UK, broken down by stage of disease.

Table 1.1 Five-year net survival by stage		
Stage	**TNM stage**	**5 year Survival (%)**
I	T1-2 N0 M0	91.7
II	T3-4 N0 M0	84.1
III	T1-4 N1+ M0	64.9
IV	T1-4 N0-2 M1	10.3

Table 1.1. Five year net survival by stage, UK, 2013-2017. Table adapted from CRUK 2018. [12]

Although these two staging systems break down survival by stage of disease appropriately, there is variation within each stage. As displayed in Table 1.1 there are patients with stage I disease who do not survive 5 years, whereas there are patients with stage IV disease who do. What characteristics that are not taken into account by Dukes' or TNM staging confer to disease prognosis?

1.6.2 Pathology

When analysing tumour the pathologists will report on a number of pathological features, in addition to stage, that are associated with stage of disease and prognosis.

1.6.2.1 Histological type

The majority of colorectal cancers are diagnosed as adenocarcinoma. Other types include small cell, squamous cell, adenosquamous, medullary and subtypes of adenocarcinoma including mucinous and signet ring cell carcinoma.[87]Mucinous adenocarcinomas have mucinous component>50%, and signet ring cell carcinomas are composed of at least 50% signet-ring cells. [87] Both mucinous and signet ring cell carcinoma are associated with higher stage of disease, and signet ring cell carcinomas have been shown to have poorer cancer specific survival. [134, 135]

1.6.2.2 Differentiation and tumour grade

Differentiation is for standard adenocarcinomas and is a measure of the degree of glandular formation. [89] This is commonly split into groups: well differentiated; >95% of the tumour is gland forming, moderately differentiated; 50-95% gland formation, poorly differentiated; <50% gland formation and undifferentiated; bearing no resemblance to the underlying tissue architecture. [136, 89] Based on differentiation the tumours are divided into two grades: low grade; well and moderately differentiated tumours and high grade; poorly and undifferentiated, with grading based upon the assessment of the predominant grade of differentiation. [87] Tumour grade is a stage-independent prognostic variable with high grade tumours have been reported to be more aggressive and have poorer survival. [89, 87]

1.6.2.3 Serosal / peritoneal involvement

Serosal or peritoneal involvement occurs when the tumour breaches the visceral peritoneum, with evidence of extension through the subserosal area. As discussed above in TNM staging, this would

categorise tumours as T4. T4 disease is known to be associated with poor prognosis, and it has been shown that T4 (stage II) disease can carry a similar or poorer prognosis to N2 (stage III) disease. [137, 138]

1.6.2.4 Margin involvement

Margins of resection are analysed in the surgical resection. As discussed above, the circumferential margin is a factor involved in the tumour component of TNM staging. Longitudinal margin involvement is also assessed. Tumour resection is defined as R0; completely excised, R1; microscopic involvement (within 1mm from resection margin) and R2; macroscopic visible tumour at the margin. [139] R1 and R2 disease is associated with local disease recurrence and poorer survival. [140]

1.6.2.5 Venous invasion

Venous invasion is defined as tumour present within an extramural endothelium-lined space that is either surrounded by a rim of muscle or contains red blood cells. [133] Extramural venous invasion is associated with significantly poorer prognosis and is an independent prognostic factor in CRC. [141, 87] Whereas the prognostic value of intramural venous invasion is less clear, a 2018 meta-analysis showed that intramural venous invasion was associated with a decreased cancer specific survival and other studies reporting no significant difference. [142, 143]

1.6.2.6 Tumour necrosis

Tumour necrosis is thought to be a result of chronic ischaemic injury. [144] It has also been linked to a high systemic inflammatory response and local inflammatory cell infiltrate. [145] The presence of tumour necrosis has been reported to be associated with poorer prognosis and cancer specific survival. [145]

1.6.2.7 Tumour Stroma Percentage

The presence of a higher stromal to epithelial volume within the tumour, also termed tumour stroma percentage (TSP). Excluding necrosis and mucin deposits, TSP is calculated as low (<50% of tumour area) or high (>50% of tumour area). A higher TSP is associated with the presence of immune-suppressive pro-cancer inflammation, advanced T and N stage. It has been shown to be a stage independent marker of reduced survival in patients with operable CRC. [146-149]

1.6.2.8 Tumour budding

Tumour budding is defined as the presence of single tumour cells or small clusters of cells within the tumour centre or at the tumour-invasion front. [150, 151] It has been associated with the tumour microenvironment .[152] Tumour budding is recognised as an adverse prognostic factor in CRC. [150, 153, 152] In fact a 1993 study showed that 5 year survival of Dukes' B patients with moderate/severe tumour budding was worse than that of Dukes' C patients with no/mild tumour budding (29.1% vs. 66.2% ($P < 0.001$). [154]

A number of other pathological features have been shown to be associated with prognosis in CRC including lymph node number, and perineural invasion.

1.6.2.9 Petersen prognostic index

In an attempt to consolidate the prognostic effects of pathological factors a number of scores have been developed. The Petersen index (PI), displayed in Figure 1.5, is a score based on four pathology features: vascular invasion, peritoneal involvement, margin involvement and tumour perforation. PI is scored from 0 to 5, with 1 point for peritoneal involvement +/- ulceration, 1 point for extramural or submucosal venous spread, 1 point if margin involved or inflamed and 2 points if perforation through tumour. [155, 9] The Petersen index has been reported to predict cancer-specific outcome in Dukes' B and C colorectal cancer. [9, 156, 157]This score gives additional prognostic information in patients with stage II CRC, who are high risk of recurrence and can be used to guide adjuvant treatment.

PI score	Total patients	Patients dying from cancer	5 year survival (95% CI)
0	82	6	94.2% (85.0–97.8)
1	109	21	79.5% (69.9–86.3)
2	63	28	54.3% (40.3–66.3)
⩾3	14	8	30.4% (7.8 – 57.4)
Total	268	63	76.1% (70.0–81.0)

Figure 1.5. Petersen Index and Survival.
Figure from Peterson et al 2002, used with permission. [9]

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1.6.3 Molecular characteristic

In recent years much work and progress has been made in understanding the complex molecular genetics of CRC. A variety of molecular markers are being studied as potential prognostic markers, but currently only a few are used in clinical practice: CEA, K-ras, and MSI.

1.6.3.1 Carcino-embryonic antigen

CEA has been used as a tumour marker for CRC. It is a complex intracellular glycoprotein that is not present in normal colonic mucosa, but is seen in 90% of CRCs. CEA has not been shown to be of value in diagnosing early CRC. However high preoperative concentrations of CEA may correlate with adverse prognosis and serial use of CEA to follow up patient's post-surgical resection allows for early detection of recurrence, and is routinely carried out in clinical practice. [115, 116]

1.6.3.2 K-ras

K-ras (Kirsten rat sarcoma viral oncogene homolog) is a member of the RAS family. Mutations in the K-ras oncogene (codons 12 and 13) lead to activated K-ras protein and uncontrolled cell proliferation. Mutations have been found in between 30-60% of CRCs. Epidermal growth factor receptor (EGFR) is an oncogene, which has oncological therapies, but tumours with K-ras mutation are resistant to EGFR targeted therapy. [87-90]

1.6.3.3 Microsatellite Instability

Microsatellite instability (MSI) is change in the number of repeats or length (due to nucleotide insertions or deletions) of normal segments of DNA with repeat sequences of nucleotides of set length. MSI is caused by defective dMMR genes, and occurs in approximately 15% of CRCs. This defective dMMR is caused by germline mutational inactivation of genes encoding MMR proteins. [89, 87] MSI is a prognostic factor in CRC, with MSI tumours being predominantly right sided, poorly differentiated with higher survival rates and respond poorly to 5-fluorouracil-based adjuvant chemotherapy. [89, 92-95]

1.6.3.4 Other molecular markers

Several other molecular markers are being studied in relation to CRC including p53 mutation, indices of cellular proliferation (Ki67), carbohydrate antigen 19-9 (Ca 19-9), matrix metalloproteinases (MMP), and BRAF gene mutation.

1.6.4 Peritumoural inflammation assessment

As I will discuss in the following chapter, inflammation plays an important role in the prognosis of colorectal cancer. A number of inflammatory based prognostic scores have been developed taking into consideration peritumoural inflammation.

1.6.4.1 Jass Criteria

In the 1980s Jass et al described one of the first assessments of the tumour inflammatory cell infiltrate. They used the knowledge that a pronounced lymphocytic infiltration in CRC was associated with a survival benefit, to score the peritumoural lymphocytic infiltrate in rectal cancer.

This was developed into a five point score. This score is displayed in Figure 1.6 with scoring one point for growth beyond the bowel wall, one point for an infiltrating invasive margin, one point for no peritumoural lymphocytic infiltrate and one to two points for the number of nodal metastasis. This scoring was extrapolated into 4 groups. [158, 7]

The Jass criteria is recognised as an independent prognostic factor of CRC cancer specific survival. [159, 160] Jass criteria has not become part of routine clinical practice.

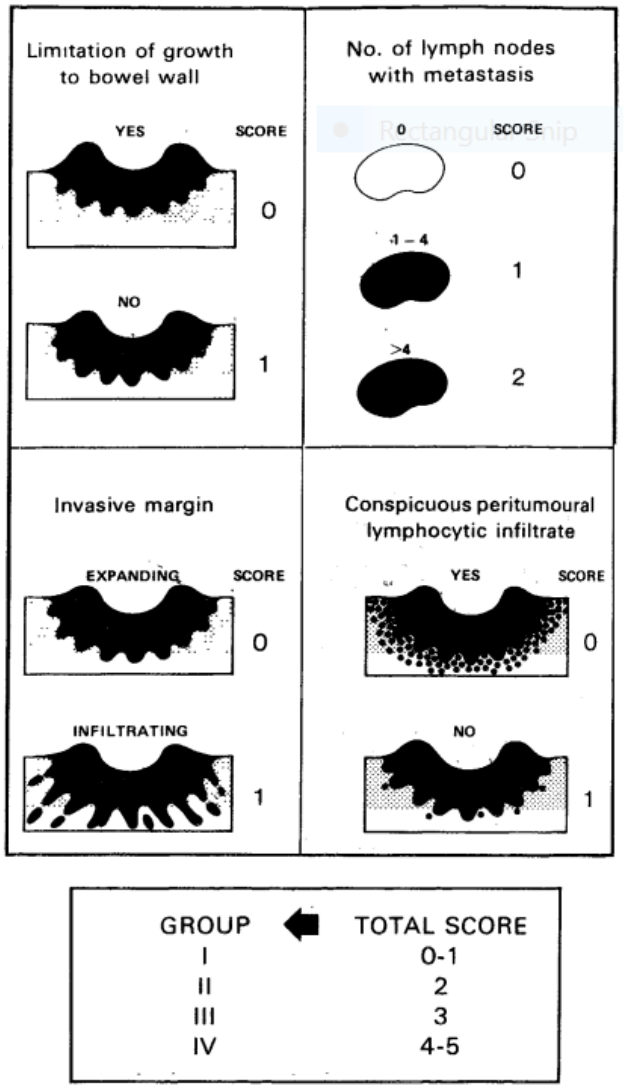

Figure 1.6. Jass Criteria.
Figure from Jass et al 1987, used with permission. [7]

1.6.4.2 Klintrup-Mäkinen Grade

Klintrup-Mäkinen (KM) grade is a semi-quantitative assessment of the peritumoural inflammatory cell infiltrate haematoxylin and eosin (H&E) stained slides. It describes the density of inflammatory cells (overall inflammatory reaction, amount of lymphoid cells, neutrophilic and eosinophilic granulocytes) at both the tumour centre and the invasive margin (interface between the host stroma and the invading edge area of a tumour). A KM score of 0-3 is determined: 0; no increase of inflammatory cells, 1; denoted mild and patchy increase of inflammatory cells at the invasive margin, but no destruction of invading cancer cell islets by the inflammatory cells, 2; inflammatory cells formed a band-like infiltrate at the invasive margin with some destruction of cancer cell islets by inflammatory cells, and 3; very prominent inflammatory reaction, forming a cup-like zone at the invasive margin, and destruction of cancer cell islets was frequent and invariably present. This is then split into low and high KM grade. [161] A high KM grade is an independent prognostic factor for improved cancer specific survival in CRC. [162, 159, 163]

1.6.4.3 The Immunoscore

Immunoscore is a scoring system which utilises immunohistochemistry to summarise the density of CD3+ and CD8+ T-cell both within the tumour centre and at its invasive margin, giving four parameters for scoring. One point is assigned to each parameter scoring if it has a high infiltrate, these points are combined to give an overall score of 0 – 4. [164, 165] This score has been validated internationally. A high Immunoscore is associated with a reduced risk of recurrence, and improved disease-free and overall survival. [164, 166-168]

1.6.4.4 Glasgow Microenvironment Score

The Glasgow microenvironment score (GMS) was created based on the prognostic value of KM grade and TSP. [1] KM relationship with survival has been explained above. The presence of a high TSP has been shown to be a stage independent marker of reduced survival in patients with operable CRC. [146] This cumulative score assesses patients with primary operable colorectal cancer. Patients fall into one of three groups: GMS 0; strong KM grade and high or low TSP, GMS 1; weak KM grade and low TSP, and GMS; weak KM grade and high TSP. [1] 5-year survival by GMS is displayed in Figure 1.7 showing survival decreased from 89% in GMS 0 to 51% in GMS 2, and GMS has now been validated as an independent prognostic tool for patients with stage I-III CRC. [1, 169]

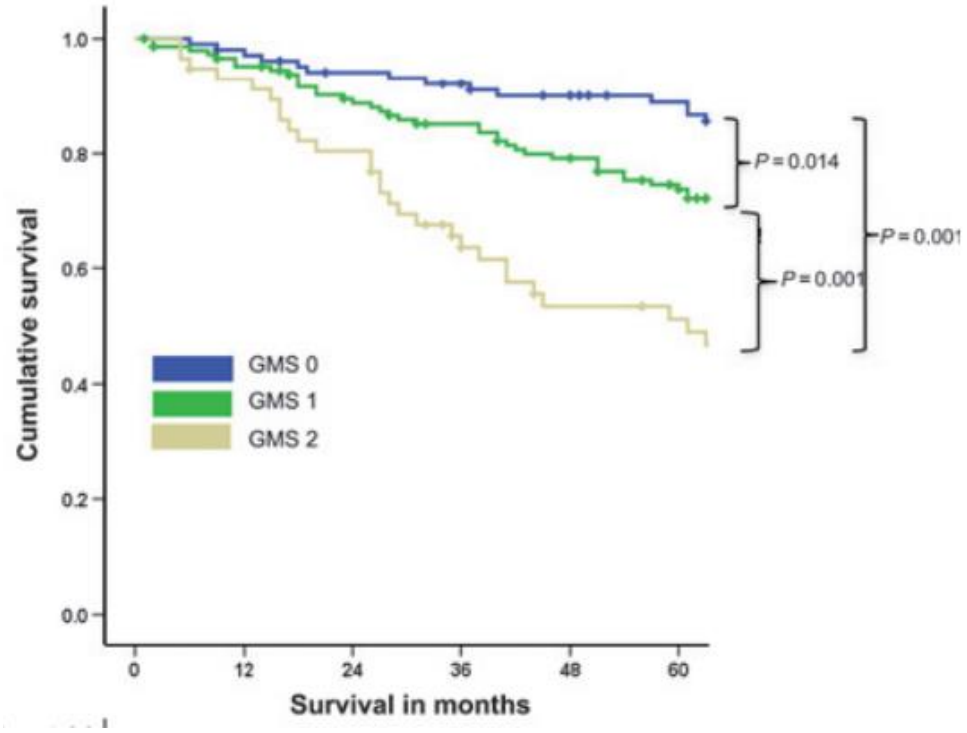

Figure 1.7. Survival stratified by GMS.
Figure from Park et al 2010, used with permission. [1]

1.7 Inflammation

1.7.1 Cancer and inflammation

Tumour suppression and oncogenic pathway activation are required for cancer to develop. This has been thought to occur secondary to a combination of environmental factors and errors in DNA repair and replication. [5] The relationship between inflammation and cancer was first brought about by Rudolf Virchow in the mid-19th century. At that time it was noted that cancer originated in sites of chronic inflammation, with abundant inflammatory cells in tumour biopsies. [170, 171, 137, 172] The relationship between cancer and inflammation is now well recognised, with the presence of an inflammatory tumour microenvironment (TME) now the seventh hallmark of cancer. [173]

1.7.1.1 Host immune system

Inflammation involving the immune system which is divided into two subsystems; the innate and adaptive immune systems. [174] The main distinction between these subsystems is the receptor types used to recognise pathogens. [175] The innate (or non-specific) immune response is mediated by pattern recognition receptors (PRRs), with targets referred to as pathogen-associated molecular patterns (PAMPs). Innate host-defence module including phagocytes, inflammasomes, natural killer (NK) cells, eosinophils and basophils. Pathogens activate these cell types through the PRRs, with release of antimicrobial proteins, chemokines, inflammatory cytokines including IL-6, Il-1ß, tumour necrosis factor (TNF) which manage an inflammatory responses. [175]

Figure 1.8 displays the adaptive immune system (or specific or acquired) which is mediated by the T-cell and B-cell antigen receptors.[175] Conventional lymphocytes and innate-like lymphocytes both express the antigens. Lymphocytes circulate until they encounter an antigen that they are specific for, with conventional lymphocytes differentiating into several types of effector cell, depending on the pathogen. The differentiation is regulated by cytokines and chemokines via the innate immune system. [175] Following activation the B-cells produce antibodies, against the specific antigens. This binding inactivates viruses and microbial toxins, marks pathogens for destruction by phagocytosis, activates the innate immune response and the complement cascade. [176, 177] There is also a cell-mediated immune response, where activated T cells react directly against

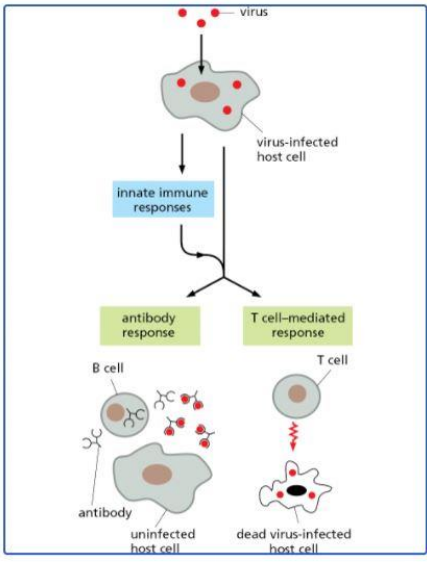

Figure 1.8. Adaptive immune response.
Figure from MOLECULAR BIOLOGY OF THE CELL 7E by Bruce Alberts, et al. Copyright © 2022 by Bruce Alberts, Rebecca Heald, Alexander Johnson, David Morgan, Martin Raff, Keith Roberts, Peter Walter, John Wilson, Tim Hunt, and the Estate of Julian Lewis. Used by permission of W. W. Norton & Company, Inc. [4]

the foreign antigen. The subset of T cells involved include cytotoxic T cells (CD8+), helper T cells (CD4+), antigen memory T cells (CD45R0+), and regulatory T-cells (FOXP3+). [177]

In cancer inflammation affects the composition of the tumour microenvironment (TME) and it was previously thought that the innate and adaptive immune systems were pro-tumourigenic and anti-tumourigenic. However it is a complex balance of the immune systems which gives anti-tumourigenic and pro-tumourigenic results. [170, 172]

1.7.1.2 Pro-tumour inflammation

The tumour-promoting role of the immune system and inflammation is complex and still not fully understood. Inflammation predisposes to the development of cancer and promotes tumourigenesis. [5] In general, the innate immune system activation triggers secretion of inflammatory, regenerative, and anti-inflammatory cytokines, causing and aiding tumour development. [178]

Figure 1.9 displays the multitude of reasons for the aetiology of cancer causing inflammation, but it is not always clear exactly how this inflammation is initiated. The immune response is responsible for the response to infection, and evolutionarily this is a more important survival tool than anti-tumourigenesis. Homeostatic inflammatory responses to inflamed tissues therefore are balanced to react to inflammatory stressors. Acute inflammation promotes maturation and function of tissue-associated macrophages and dendritic cells (DCs), recruitment and amplifications of immune cells all of which also promote tumourigenesis. [5, 174] This type of inflammation is usually resolved once the stressor i.e. infection has been removed. However the inflammatory response during cancer development is non-resolving. [174, 179] This inflammation can be caused by host and

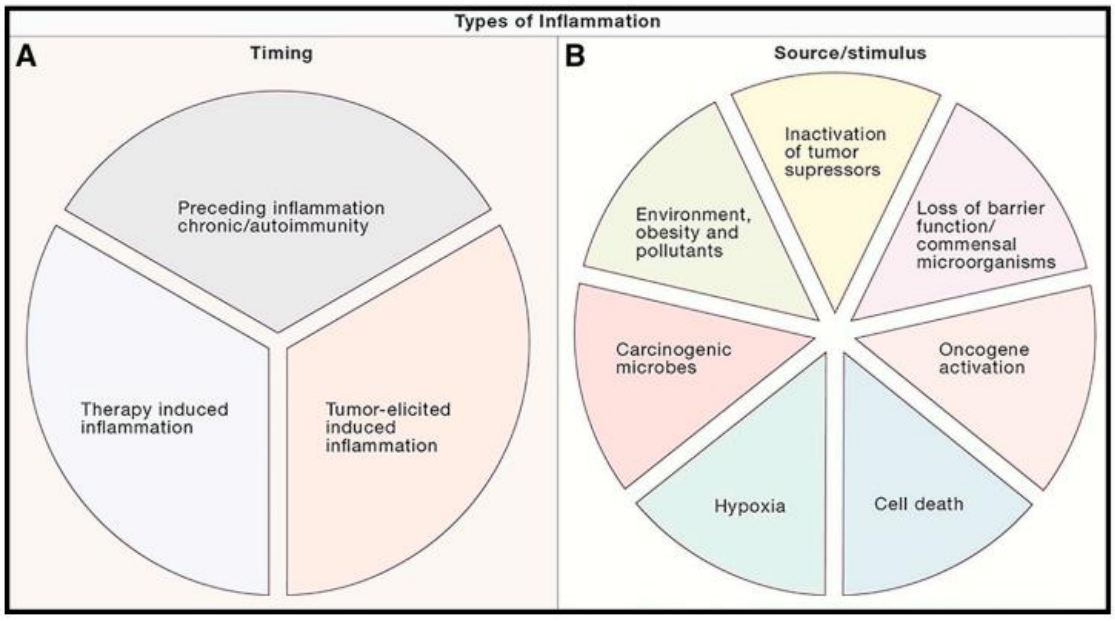

Figure 1.9. Aetiology of cancer inflammation.
Figure from Greten et al 2019, used with permission. [5]

environmental factors such as chronic infections, obesity, smoking, alcohol consumption, or by an oncogenic event. [174] Around one fifth of all cancers are preceded by chronic inflammation at the site of the cancer e.g. colon cancers in IBD, oesophageal cancer in Barrett's oesophagus. [172, 5]

However the majority of cancers develop in areas with no preceding chronic inflammation. Therefore tumours recruit immune cells to aide growth termed tumour-elicited inflammation. [5] This may be triggered from sensing oncogenic events from metabolic alterations, cell death or hypoxia. [180] Tumour cell death by necrosis may have immunostimulatory effects. An inflammatory response would stimulate the production of cytokines and growth factors. These may cause anti-apoptotic signals. [5]

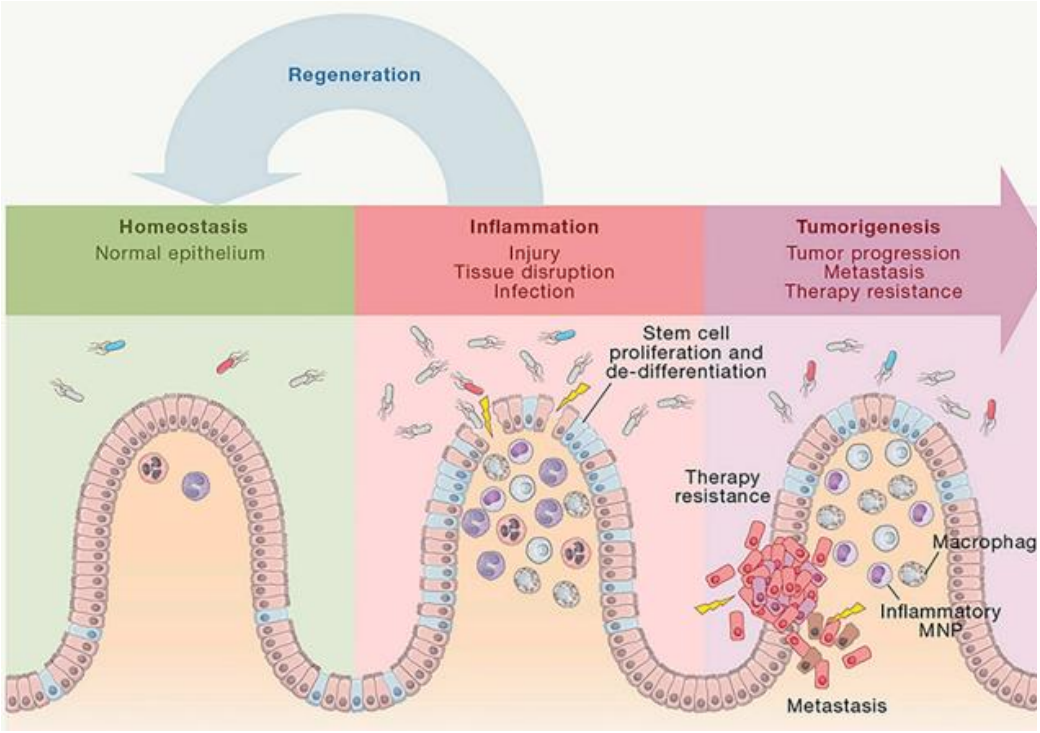

Figure 1.10. Normal tissue to tumourigenesis.
Figure from Greten et al 2019, used with permission. [5]

A number of proinflammatory mechanisms are thought to drive cancer associated inflammation. Figure 1.10 displays the process from normal tissue to cancer by inflammation. Tumour suppressive mechanism are usurped in cancer. Loss of tumour suppression causes loss of usual DNA damage surveillance, inhibiting DNA repair and results in DNA damage. Mutations of tumour suppressors occur including Tp53. Tp53 encodes for P53, a transcriptional antagonist with nuclear factor-kappa B (NF-κB), a positive regulator of inflammation. NF-κB promotes tumour cells proliferation, suppresses apoptosis, attracts angiogenesis, and induces epithelial mesenchymal transition, facilitating distant metastasis. [5, 181] Activation of oncogenes is linked to increased production of cytokine and chemokines. Mutations of oncogenes e.g. the K-ras oncogene (regulates

production of cytokines and chemokines), leads to excessive production of the inflammatory entities, and uncontrolled cell proliferation. [5, 87-90] Inflammatory cells such as macrophages and neutrophils produce reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) species, which induce mutations. [5] At the early stage of tumour development, a full tumour microenvironment (TME) has not developed, but inflammation and injury triggers inflammatory cytokines which cause cell turnover and tumour initiation.[5]

As well as triggering cytokines, Figure 1.11 shows how inflammatory cells modulate the TME to promote tumour growth by stimulation of angiogenesis, recruitment of stromal cells, and causing immunosuppression. [5, 172] Recruitment of myeloid cells facilitates cell migration, and myeloid derived suppressor cells (MDSC) contribute to the suppression of anti-tumour responses.

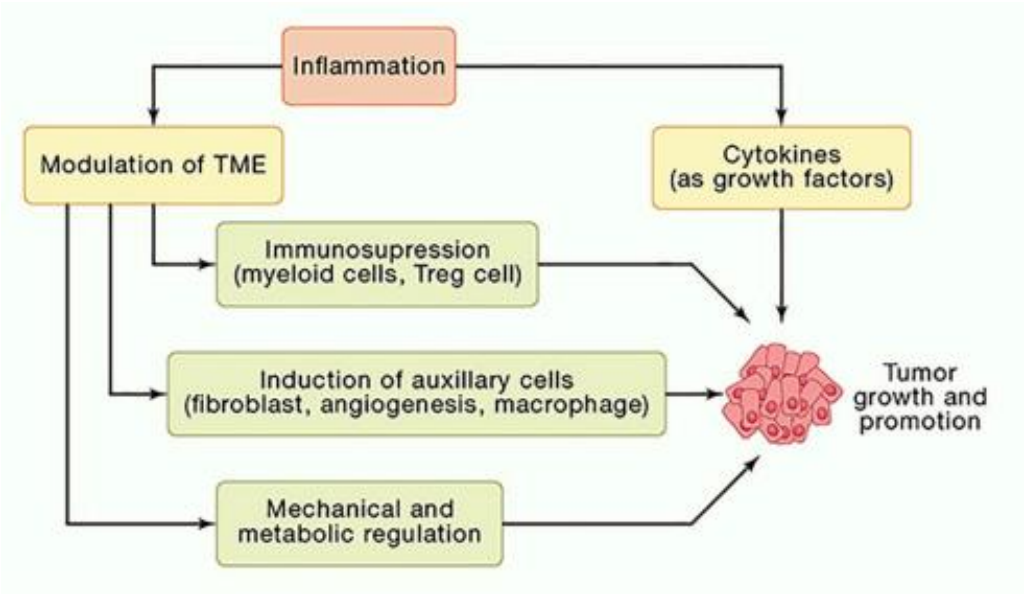

Figure 1.11. TME modulation.
Figure from Greten et al 2019, used with permission. [5]

There is considerable tumour heterogeneity in the TME, with differences in characteristics of immune and stromal cells. T cells and B cells represent the adaptive immunity. B cells regulate anti-cancer immune responses, exert direct tumour promotion and modulate myeloid cell response as determined by the TME.T cells, in the TME, acquire tumour promoting functions associated with cytokine expression. [5]

1.7.1.3 Anti-tumour inflammation

Anti-tumour immunity is a multifaceted process requiring T cell priming against tumour antigens, transportation of the anti-tumour T cells to the tumour, T cell infiltration of the tumour and local activation to kill tumour cells. Tumour antigenicity is driven by mutations resulting in tumour neoantigens. [182]

Inflammation is a protective response to injury and infection, with the immune response divided into three phases. The final, resolution, phase is characterised by production of anti-inflammatory lipid mediators and cytokines, which are involved in termination of damaging inflammation and initiation of cell repair and regeneration. They also enhance the adaptive immune system, increasing the regulatory T and B cells that suppress immune activation. [178] Chronic inflammation is also associated with production of TGFβ, which supports differentiation of immunosuppressive cells including regulatory T reg. [178] Vascular endothelial growth factor (VEGF) is involved in the formation of new blood vessels during the resolution and healing phase of inflammation. [178] Tumours secrete cytokines, and a pro-inflammatory cytokine, IL-6. IL-6 promotes Th cell differentiation, effector functions and naïve CD8+ T cells. [178]

The TME consists of T cells, myeloid cells, and fibroblasts that modulate T cell trafficking and activation. [182] The anti-tumour immune response is initiated by T cell priming, mediated by antigen presenting cells. Tumour antigens are detected by antigen presenting cells, for T cell modulation. Interaction and activation of T lymphocytes targets the key tumour cells. [183] These signals are induced by inflammation, to help enhance their expression. [178] This involvement of the adaptive immune system in anti-tumour immunity is affected by intrinsic tumour characteristics, microenvironment factors, and genetic/epigenetic determinants resulting in tumour heterogeneity .[183]

Overall anti-tumour and pro-tumour cancer immunity balance is a highly complex, intertwined, balance of immunity. Minor alterations and changes can disrupt an anti-tumour immune response resulting in a pro-tumour response. [183]

1.7.2 Systemic Inflammatory Response in CRC

The SIR is a physiological response to tissue injury or pathogens, occurring distant to the original site of inflammation [184] A large number of changes occur as a response to inflammation, referred to as the acute phase response. [184]

Changes occur in the concentrations of the acute-phase proteins (e.g. complement system, CRP, albumin, ferritin, fibrinogen) as well as behavioural, physiologic, biochemical, and nutritional changes. Macrophages and monocytes at inflammatory sites produce cytokines. Cytokines e.g. IL-6, IL-1ß, TNF, are produced during inflammation, and work as a cascade and network to stimulate the production of acute-phase proteins. Patterns of cytokine production and the acute-phase response differ in different inflammatory conditions. Inflammation-associated cytokines have been implicated in the pathogenesis of anaemia in chronic disease, thrombocytosis and cachexia. [184]

CRP is one of the acute phase proteins, and levels rise in response to inflammation. It is probably the most measured acute phase protein in clinical practice. It is induced by the IL-6 action on the gene responsible for CRP transcription. CRP has pro-inflammatory and anti-inflammatory properties. It recognises pathogens, induces phagocytosis, and activates the complement cascade. However it can have a negative effect by this activation of the complement cascade and subsequent release of inflammatory cytokines. [185]

The relationship between the presence of a SIR, colorectal cancer development and outcomes is now well described. Systemic inflammation measured in peripheral blood is a validated, stage-independent predictor of poorer cancer outcomes in both early resectable colorectal cancer as well as advanced disease. [186, 187] In 2003 McMillan et al described how the presence of a SIR, in patients who had undergone a potentially curative resection for CRC, predicted a poor outcome. CRP levels pre operatively were significantly associated with overall and cancer-specific survival. [52] A comparison of measures or components of the SIR, found that modified Glasgow prognostic score (mGPS) was independently associated with cancer-specific survival and that mGPS, appears to be a superior component of the SIR to predict survival. [188]

1.7.3 Systemic inflammation assessment

A number of inflammatory based prognostic scores are used, and have been developed, to assess patients' SIR.

1.7.3.1 Modified Glasgow prognostic score

The modified Glasgow prognostic score (mGPS) is a measure of the systemic inflammatory response (SIR). It combines preoperative values of serum C-reactive protein (CRP) and albumin. Patients are scored from 0-2: 0; CRP ≤10mg/l and albumin ≥35g/l, 1; CRP>10mg/l, 2; both CRP >10 mg/l and albumin<35 g/l. [189] mGPS is an independent prognostic factor in CRC. [189-192]

In CRC, the role of both local colonic inflammation, as well as systemic inflammation can help determine cancer progression and survival. [193, 147, 194]

1.7.3.2 Neutrophil lymphocyte ratio

Neutrophil lymphocyte ratio (NLR) is an inflammatory biomarker calculated by dividing the absolute number of neutrophils by the absolute number of lymphocytes, to display neutrophilia accompanied by a relative lymphocytopaenia in SIR. [195] NLR thresholds vary, with NLR >5 being associated with reduced 5 year overall and cancer specific survival in CRC. [196] More recently an NLR more than the median of ~2.74 is reported to be associated with reduced disease specific survival. [197]

1.7.3.3 Platelet lymphocyte ratio

Platelet lymphocyte ratio (PLR) is a prognostic score similar to NLR, because it is based on components of the differential white cell count but using platelets rather than neutrophils. It was first reported as a prognostic marker in cancer in 2009, when preoperative PLR was shown to be independent prognostic marker in patients with resected pancreatic adenocarcinoma. In CRC it has been shown that PLR correlated significantly with positive lymph node ratio, and a PLR of > 300 was an independent prognostic factor of overall survival. [198, 199]

1.7.4 Local Inflammatory Response in CRC

As discussed in section 1.7.1, within the tumour microenvironment, conflicting pro-tumour and anti-tumour local inflammatory responses (LIR) can dictate cancer outcomes.

Both the presence of a generalised inflammatory cell infiltrate (both adaptive and innate immune cells), and specific immune cell types have been shown to be associated with a good prognosis. Assessment of the LIR via inflammatory scores has been described in 1.6.4 including KM grade, The Immunoscore and GMS.

A pronounced local lymphocytic inflammatory infiltrate within the tumour microenvironment is associated with an improved prognosis in primary resectable colorectal cancer.[193, 147, 194]. Jass et al described the assessment of peritumoural lymphocytic infiltrate, a stage independent marker of cancer specific survival in CRC (see 1.6.4.1). The Crohn's like reaction describes aggregates of lymphocytes around the tumour which were associated with improved prognosis in colorectal cancer. [200]

Tumour-infiltrating lymphocytes (TILs) are an important prognostic feature of CRC, being significantly associated with cancer specific and overall survival. Studies show that high levels of generalized TILs have an improved overall survival. [201] Specific immune cell types including subsets of T-lymphocytes: CD3+, CD8+ cytotoxic, CD45RO+ memory, FOXP3+, tumour associated macrophages (TAMs), dendritic cells and neutrophils, also relate to recurrence and survival. [202, 193]

Higher CD3+ T-lymphocyte infiltration is associated with lower T and N stage, reduced recurrence and improved survival. Lower CD3+ T-lymphocyte infiltration is associated with the development of distant metastases. [203, 193, 202] Higher CD8+ T-lymphocyte infiltration is associated with improved survival. [193, 201] Increased density of CD45RO+ memory T lymphocytes within the TME is associated with decreased lymphatic, perineural and venous invasion as well as a better prognosis. [203] FOXP3 is a T regulatory cell marker. FOXP3+ T-cells at the tumour centre are thought to be associated with improved prognosis for cancer-specific survival and overall survival, but studies have shown mixed results and the converse prognosis in other cancer types. Recent studies have suggested that FOXP3+ has multiple subtypes, which may explain the variability. [202, 204] Increasing density of TAMs, (particularly at the tumour margin) was independently associated with survival and associated with absence of metastases, as well as no lymph node or distant metastases. [193, 205]

Overall the LIR is a prognostic indicator of higher stage disease and survival in CRC. There are numerous cell types and scoring systems to assess the LIR, however to date none of these are assessed in routine clinical practice.

1.8 Faecal calprotectin

The presence of an inflammatory based microenvironment is a key hallmark of cancer. [173] Colonic inflammation drives carcinogenesis and is a prognostic indicator for disease progression and survival in CRC. [193, 147, 194] Inflammatory parameters measured at both systemic and peritumoural level are important in determining progression and outcome in CRC. Faecal calprotectin (FC), a measure of colonic inflammation, represents another assessment of inflammation. Whether colonic inflammation measured by FC has a role in development or progression of CRC is not known. Furthermore, it is unclear whether existing measures of local and systemic inflammation relate to colonic inflammation measured by FC.

1.8.1 Faecal calprotectin

Calprotectin detected in the faeces is one sensitive measure of colonic inflammation used mainly in the clinical assessment of IBD. [206] Calprotectin belongs to the S-100 protein family, consisting of three polypeptide chains and is found predominantly in the cytoplasm of neutrophils and the membrane of monocyte. [206-208] Calprotectin is released upon neutrophil cell death or damage, and is thought to have regulatory roles on components of the inflammatory process including other myeloid derived cells (e.g. CD11b+ cells). [206, 209, 210] Calprotectin may also play a role in inducing apoptosis, as studies have shown that calprotectin inhibited the growth of several human cell lines. [211, 212] Calprotectin enters the bowel lumen by migration and is resistant to enzymatic degradation and therefore can be readily detected in bodily fluids such as faeces. [207, 209, 211, 213]

1.8.2 Faecal calprotectin testing

Several tests are available in the UK for measuring FC. The most common being Enzyme-linked immunosorbent assay (ELISA), which was first used by Roseth et al. in 1992. [214] In 2000, a new assay became available which was reported as five times more sensitive in comparison to the original (µg/g rather than mg/L). [215]

Patients are asked to provide a stool sample, ideally first stool of the day, and return to their practitioner as soon as possible. Stool samples for FC, can be stored for up to 3 days, without refrigeration which allows FC to be assessed in real life clinical practice. [216-218]

The current cut-off for ‘normal’ has been defined by manufacturers at 50µg/g. [219] .Local studies suggest that, in adult patients, FC values <200µg/g are rarely associated with IBD or other significant luminal pathology. [220, 221] Local guidelines and reference range therefore deems <50µg/g is normal, but only >200µg/g is deemed clinically significant. [222]

1.8.3 The current diagnostic role of faecal calprotectin

FC is an established marker of inflammation predominantly used in diagnosis and monitoring of IBD. [206, 223]

Abdominal pain and diarrhoea are common symptoms that patients present to their doctor with. These are also potentially symptoms of IBD, however only a small percentage of these patients will be diagnosed with IBD. Many studies have shown that patients with IBD have elevated levels of FC compared to healthy patients. [224-227] Therefore FC, as a non-invasive diagnostic marker, can be used to differentiate patients who need further investigations for IBD.

Following on from a diagnosis of IBD, these patients require treatment and long-term follow-up and management. As part of this management monitoring of disease activity is required to allow appropriate adjustments to medication. This will encompass assessment of clinical symptoms as well as endoscopy and imaging. FC has been shown to correlate with level of disease activity. Roseth et al. showed a significant correlation between FC and endoscopic and histologic activity in patients with ulcerative colitis. [224] This has since been confirmed by multiple studies, reporting a correlation between FC and IBD activity, including remission of IBD. [228-230] However, the strength of this correlation is variable between studies and therefore FC is still used in conjunction with other assessments. [231] FC have been found to rise when IBD is relapsing. [232]

It is also thought that high FC levels can occur in a wide variety of other GI conditions including infective colitis and malignancy. [233, 234] However FC is not used clinically in the UK, out with the remit of IBD.

1.8.4 Future use of faecal calprotectin

FC in IBD is a clinically recognised and accepted investigation. However does FC have other potential uses?

I have discussed the faecal tests which have been used as part of bowel cancer screening and as part of the diagnosis of symptomatic patients with CRC. However as I touched upon, the optimal screening technique is not yet known. The current methods of FOBT/FIT combined with colonoscopy is sensitive, but their specificity is lacking. [235] Identifying a non-invasive investigation, with higher sensitivity and specificity, to aid in the diagnosis of CRC therefore remains highly sought after.

There are numerous cell types and scoring systems that have been studied that appropriately assess the LIR, however to date none of these are used in routine clinical practice. These could be used to identify patients at high risk of developing CRC recurrence to allow targeted early adjuvant management. These current methods all rely on analysis of a pathology specimen, which renders

the tests potentially complex and expensive to initiate which would not be appropriate for population-based screening, and may be why these scoring systems have not been adopted into current clinical practice. If FC is related to inflammation in CRC, it could be a simple measure of the LIR in CRC.

2.0 Summary and Aims

2.1 Summary

Colorectal cancer is the fourth most common cancer and the second most common cause of cancer death in the UK. Survival has improved with 5 year survival now approximately 60%. This differs between stages of disease at diagnosis, with 91% five year survival in stage 1, compared to 10% five year survival in stage IV. Even in patients who undergo planned curative resection, disease can recur in the following years. It is understood that a multitude of factors play into recurrence, survival and prognosis from CRC.

In CRC, the role of both local colonic inflammation, as well as systemic inflammation can help determine cancer progression and survival.

The presence of an inflammatory tumour microenvironment has been recognised as the seventh hallmark of cancer. The relationships between the presence of a systemic inflammatory response and colorectal cancer development is well described. Within the tumour microenvironment, conflicting pro-tumour and anti-tumour inflammatory responses can dictate cancer outcomes. The presence of a higher stromal to epithelial volume within the tumour is associated with presence of immune-suppressive pro-cancer inflammation and poorer cancer outcomes. Conversely a pronounced local inflammatory response characterised by a high grade lymphocytic infiltrate within the tumour microenvironment is associated with improved cancer outcomes.

The majority of CRCs are diagnosed at an early, potentially curative stage. However many of these cancers will later recur, some at an early stage. One focus of research in recent years has been to determine why this happens and how to identify these patients early to improve the recurrence rate. The local inflammatory response in the tumour microenvironment is one such area which may be targetable for modification, possibly at a neoadjuvant stage. Present methods of assessing the tumour microenvironment rely on tissue sampling, and are typically reliant on post-operative specimens. Despite the acknowledged prognostic relationship, assessment of the local inflammatory response is currently not a requirement in staging of CRC. This may be due to both the heterogeneity, and the technical skills required to assess LIR via current methods.

If FC does correlate with colorectal neoplasia, systemic inflammation and the tumour microenvironment it could have potential benefits including in diagnosis and staging of CRC. To further evaluate these research themes, I formulated the following hypotheses:

1. Elevated faecal calprotectin is associated with presence of colorectal cancer.

2. Elevated faecal calprotectin is associated with presence of a systemic inflammatory response.

3. Both faecal calprotectin and the systemic inflammatory response could be used alone, or in combination, to further risk stratify for presence of colorectal cancer within a bowel screening cohort.

4. Faecal calprotectin reflects increasing tumour burden reflected by more advanced disease staging more aggressive histological features.

5. Elevations in faecal calprotectin reflect a more inflammatory or immune active tumour microenvironment where colorectal cancer is present.

2.2 Aims

In order to test these hypotheses, I have formulated the following aims for this thesis in order to examine the relationship between colorectal neoplasia, faecal calprotectin and the inflammatory response:

1. Characterise the relationship between elevations of faecal calprotectin and colorectal neoplasia, in order to ascertain whether there may be any value in its routine assessment as part of the diagnostic process.

2. Determine the relationship between faecal calprotectin and the presence of colorectal neoplasia, in a cohort of FOBT positive, Scottish Bowel Screening Programme (SBSP) patients.

3. To characterise the relationship between presence of a systemic inflammatory response and luminal inflammation measured using faecal calprotectin with a bowel screening cohort.

4. To evaluate whether presence of systemic inflammation, in addition to measurement of faecal calprotectin can provide additional discriminatory information to identify patients with colorectal neoplasia with a bowel screening cohort.

5. To examine the relationship between FC measurement and individual staging and pathological characteristics in colorectal cancer.

6. To examine the relationship between FC and elements of the local tumour inflammatory response in patients with colorectal cancer.

2.2.1 Funding

All of the studies in this thesis were conducted with funding provided by the Academic Unit of Surgery at Glasgow Royal Infirmary.

3.0 The role of faecal calprotectin in diagnosis and staging of colorectal neoplasia: A systematic review and meta-analysis

3.1 Introduction

CRC remains a common cause of cancer and cancer death in Scotland. [13, 14] Population-based bowel cancer screening programs have been implemented globally with the aim of clearing premalignant lesions and detecting CRC at an earlier stage to improve overall CRC mortality. [236] The optimal screening technique is not yet known and while current methods including FOBT and FIT combined with colonoscopy appear sensitive, their specificity is lacking. [235] Colonoscopy is an invasive, expensive test. Strategies to improve current diagnostic and screening models would be beneficial. Improving the sensitivity and specificity of non-invasive investigations in diagnosis of CRC is therefore highly sought after. To help aide the identification of the highest risk patients, who would benefit the most from further investigations.

Colonic inflammation can drive carcinogenesis and the presence of an inflammatory based microenvironment is a key hallmark of cancer. [173] Furthermore, the role of inflammatory responses at a local and systemic level, play important roles in disease progression and survival in CRC. [193, 147, 194] To date most work has focussed on local characterisation of the immune cell/ inflammatory make-up within established CRCs. Broadly speaking, it is apparent that adaptive (T-cell rich) responses are associated with improved outcomes whereas innate (myeloid derived) responses may have more pro-tumour effects. [237, 238, 193] Current methods of assessment of local inflammation rely on tissue sampling which may not be appropriate for population-based screening.

Calprotectin detected in the faeces is one sensitive measure of colonic inflammation. Elevations of FC occur in a wide variety of gastrointestinal (GI) conditions including colitis and malignancy and although a sensitive measure of inflammation, it is not specific for any single condition. Nonetheless, given the importance of inflammation in cancer development and progression, the presence of an elevated FC may provide additional discrimination of a patient's risk of colorectal neoplasia and progression. I discuss faecal calprotectin; its makeup, role and current clinical significance in more detail in chapter 1.8.

The role of FC as a diagnostic test that may categorise patients by risk of neoplasia (adenomas and carcinomas) is poorly defined. Furthermore, it is not clear whether FC values show any correlation with tumour characteristics including disease stage or location. The aim of this systematic review and meta-analysis is to attempt to characterise the relationship between elevations of FC and colorectal neoplasia, in order to ascertain whether there may be any value in its routine assessment as part of the diagnostic process.

3.2 Methods

A systematic review was performed with the aim primarily to define the relationship between FC and presence of colorectal neoplasia and secondarily whether FC can be used to aid staging of colorectal cancer. Review methodology followed the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) statement. [239]

3.2.1 Eligibility Criteria

To be included in this review, the studies had to examine either FC in relation to colorectal neoplasia (including cancer) or in relation to stage of colorectal cancer, in human studies of participants aged >18 years. Studies looking at the relationship of FC to other pathologies (not including cancer), animal, children, pre-clinical, non-English, duplicates and abstract-only studies were excluded.

3.2.2 Information Sources

A literature search was made of the US National Library of Medicine (MEDLINE, via PubMed), the Cochrane Database of Systematic Reviews and Ovid. Search was performed from inception to 31 March 2017. The search was later extended to 31 March 2021. The bibliographies of relevant studies were hand-searched for any additional relevant studies to be included.

3.2.3 Search Strategy

The following search terms were used "calprotectin AND (neoplasia OR malignancy OR cancer)". These final search terms were chosen after a number of provisional searches because they returned the greatest number of relevant abstracts to the review topic.

3.2.4 Selection Process

The titles and abstracts of all the studies returned by the search terms were reviewed. The full text of studies not excluded at this stage were obtained and reviewed, to determine if they meet the inclusion and exclusion criteria. The selected were studies were grouped into 'FC in colorectal neoplasia' and 'FC in different stages of CRC'. Four of the included papers investigated both FC in colorectal neoplasia, as well as in different stages of CRC. This selection process was performed by one researcher (FR). This selection process is summarised in the flow chart (Figure 3.1).

3.2.5 Data Collection and synthesis

A standardised form was used for recording data extraction and collection for each paper. This encompassed paper details including author and year, whether the paper met the required criteria, sample size, indication for FC in patients, how FC was assessed and what measurement and cut-off of FC was used. Sensitivity and specificity data was retrieved directly from the studies. In some studies the raw data was given, and this enabled this data to be calculated. Meta-analysis (random effect model) of FC levels in colorectal neoplasia (adenomas, advanced adenomas and CRC) was

undertaken. Values were again extracted directly from the studies or calculated from the values given.

Data for colorectal neoplasia was assessed in the form of adenomas, advanced adenomas and colorectal cancer. Adenomas were considered advanced adenomas if they were >10mm in size, had severe dysplasia or villous components. Adenomas without these features were grouped together and termed low-risk.

Several tests are available in the UK for measuring FC. The Enzyme-linked immunosorbent assay (ELISA), a newer assay with reported as being five times more sensitive in comparison to the original (μg/g rather than mg/L). [215] Old and new results can be directly compared by simply multiplying the former by a factor of 5. [215] To allow comparison of the two main units these have all been standardised, for this review, by converting mg/l to μg/g as described above.

3.2.6 Risk of bias assessment

Bias was recorded as unclear risk for all studies. Overall bias has been illustrated in funnel plots in Figures 3.4a-c.

3.2.7 Statistical Analysis

Data analysis was performed using Review Manager (RevMan) Version 5.3 (The Nordic Cochrane Centre, The Cochrane Collaboration, Copenhagen, 2014). $p < 0.05$ was considered significant and heterogeneity was assessed with the I2 test. Forest plots were created to display the study results, with the overall odds ratio (OR) and 95% confidence interval. Sensitivity and specificity, positive predictive value (PPV) and negative predictive value (NPV) calculations was performed manually.

3.3 Results

3.3.1 Study Selection Process

The selection process is summarised in Figure 3.1. Using the search protocol described in the methods, 2357 papers were found. The titles and/or abstracts for these were reviewed and 1980 were excluded based on abstract review. Full text analysis was completed for 102 papers and 32 were included for the purposes of this review. The reference lists of the final papers were hand searched and three additional relevant papers were included. Thirty five papers were included in total. Four of the included papers investigated both FC in colorectal neoplasia, as well as in different stages of CRC.

3.3.2 Units for faecal calprotectin and cut-off points

Several tests are available in the UK for measuring FC. The most common being ELISA. In 2000, a new assay became available measuring in μg/g rather than the previous mg/L. [215] Old and new results can be directly compared by simply multiplying the former by a factor of 5. [215] There is currently no preference for what test should be performed in the UK. [223] The current cut-off for 'normal' has been defined by manufacturers at 50μg/g, with some going further and stating that levels of ≥200μg/g signify that inflammation is present and further investigations are required. [219] Cut-off points for specific colonic diseases including neoplasia are not known.

The studies in this review cover a variety of assays and cut-off points reflecting the long time period of more than 25 years (1992 – 2021) over which FC has been analysed and the technological advances that have occurred. Table 3.1 details the testing methods and thresholds for FC.

Table 3.2 displays all of the papers in this analysis and how patients were recruited for each study.

3.3.3 Faecal Calprotectin and Adenomas

I hypothesised that FC would be lowest in patients with no colorectal pathology with a sequential rise through the stages of neoplasia.

Nineteen studies examined whether FC correlates with degree of colorectal adenomas. Five of the nineteen studies were not included in the final analysis as insufficient information was available to allow for direct comparison. Nine studies including 5350 patients reported median values for their datasets (Table 3.3). Twelve studies including 6555 patients reported sensitivity and specificity data (Table 3.4). Seven studies did not report sensitivity, specificity, PPV or NPV, but reported data that allowed for these to be calculated. Three of the eleven studies did not report specific sensitivity or specificity figures, or allow for these figures to be calculated. [240-242] However, significance of results was reported therefore the studies have been included in this review.

Seven out of nine studies showed median FC levels were higher in adenomas and in turn colorectal cancer, in comparison to normal patients. Six out of seven studies reported higher median FC levels specifically in adenomas compared to patients with no colorectal pathology. All nine studies reported lower levels of FC in adenomas or advanced adenomas in comparison to colorectal cancer.

The sensitivity and specificity of FC for both adenomas and advanced adenomas covered a wide range. For adenomas a sensitivity ranged from 28.0-56.2% and specificity 25.0-85.0%, and correspondingly in advanced adenomas 26.7-66.6% sensitivity and specificity 37.8-76.1%, using the cut-offs mentioned in the previous section. As the cut-off value for FC increased, the sensitivity for both adenomas and advanced adenomas reduced and specificity increased.

The PPV is lower than the negative predictive value (NPV) for all studies for both adenomas and advanced adenomas reflecting the sensitive (but not specific) nature of FC. For advanced adenomas in particular, the NPV was >89% in all studies and PPV was <25%. This suggests that in patients with a normal FC, it is less likely that they will have an advanced adenoma. However a high FC does not give a diagnosis of adenoma or advanced adenoma specifically. Figure 3.2a displays the five studies included in a meta-analysis of FC levels in patients with adenomas. In this small number of studies, OR ranged from 0.13 to 2.89, overall OR 0.84 (95% CI 0.31-2.22) with high heterogeneity (I^2=90%), p=0.72. Figure 3.2b shows the three studies included in meta-analysis of FC levels in patients with advanced adenoma. This showed overall OR 1.17 (95% CI 0.82-1.68), I^2 = 30%, p=0.40. This shows a lack of evidence supporting an association between FC and adenoma/advanced adenomas and confirms the variable nature of FC in relation to its use in adenomas.

3.3.4 Faecal Calprotectin and Colorectal Cancer

Thirty-four studies examined whether FC correlates with CRC. Three of the thirty-four studies were not included in the final analyses as insufficient information was available to allow for direct comparison. Twenty-two studies including 1128 patients with CRC reported median FC values for their datasets in CRC (Table 3.3). Pavlidis et al. was excluded as it only included one CRC patient. [243] Fifteen studies including 8197 patients (429 CRCs) reported sensitivity and specificity data (Table 3.5). Borza et al. was excluded from this table, because while they reported sensitivity and specificity, the comparison was cancer of diabetic patient's vs cancer of non-diabetic patients. [244] Three studies did not report sensitivity, specificity, PPV or NPV, but reported data that allowed for these to be calculated.

For healthy individuals, median calprotectin was low with range of 2.3-11.5mg/l and 10-46 μg/g. In CRC the median calprotectin was higher with range 17.6-101mg/l and 19.3-420.5μg/g. Median FC was higher in CRC in fifteen out of the sixteen studies that reported median values for both healthy and CRC subjects (with half of the studies reporting a significant difference). Only one study reported the same results for both healthy individuals and those with cancer. [245] All nine studies reporting median FC in both adenoma and CRC patients reported higher median FC in CRC compared to any degree of adenomas.

Sensitivity and specificity range from 68.0 - 100% and 35.2 – 84.0% respectively in CRC. As the cut-off for FC increases, there is a fall in sensitivity with a corresponding rise in specificity. In CRC FC (using cut off 50μg/g) has a high negative predictive value with seven out of eight studies reporting a value >95%, and all studies reporting a NPV >85%. However, this is at the detriment of a low positive predictive value with five out of eight studies reporting a value <10%.

Meta-analysis of seven studies of FC in CRC was performed (Figure 3.3). Patients with CRC are 5-fold more likely than controls to have an elevated FC (OR 5.19, 95% CI 3.12-8.62, P<0.001 with a heterogeneity (I^2=27%)).

3.3.5 Faecal Calprotectin and Staging of Colorectal Cancer

There are fewer studies reporting FC's relationship to stage of disease or tumour histopathology, in colorectal cancer. In this review eight studies incorporated various elements of this relationship.

Table 3.6 displays the seven studies reporting on FC in different stages of colorectal cancer. For comparison these have been grouped per stage of disease. Lehman et al. (2014) showed that T-stage correlated with FC with patients with T3/4 disease having significantly higher FC levels than T1/2 disease (p=0.022). [246] Kristinsson et al. (2001) reported that those with Dukes' A disease had lower FC levels, but this result was not of statistical significance. [211] This is similar to that reported by Karl et al. (2008) with median FC levels of 179.2μg/g in Dukes' A and at least >300μg/g for Dukes' B-D. [247] However no other study has shown any significant correlation between FC and stage of colorectal cancer.

Table 3.7 displays the six studies that examined whether location of colorectal cancer correlates with FC. Three of these studies looked at the difference between the right and left (but did not specify the exact definition of this). None of the studies showed any significant difference in FC based on the location of the tumour. One study looking only at colorectal neoplasia showed that patients with proximal colonic neoplasms (median 53.8μg/g) had a higher FC than those with distal neoplasms (median 23.0μg/g) (P=0.001), however it was based on a small number of cases (16 proximal, 27 distal and 54 both). [248]

One study reported no significant difference in FC between differentiation of disease. [249] Kristinsson et al. (2001) reported no significant difference in FC in grade or size of colorectal cancer. [211]

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3.4 Discussion

This systematic review and meta-analysis sought to characterise the relationship between elevation of FC and colorectal neoplasia, in order to ascertain whether there may be value in its routine measurement as part of the diagnostic or pre-operative staging process in CRC.

Ye et al published a meta-analysis on the diagnostic accuracy of FC for screening for CRC, which reported that FC cannot be recommended for CRC detection. [250] Our current review adds to this literature by aiming to define the relationship between FC and presence of colorectal neoplasia as well as how FC varies with different stages of colorectal cancer.

The potential relationship between FC and colorectal cancer has been of interest since the early 1990s when Roseth et al. [214] first published their work on the subject. Their study looked at extraction and quantification of calprotectin but found that 10 out of 11 patients with GI cancers had an elevated FC. [214] This was followed by a pilot study. It showed that 94% of CRC patients had elevated FC levels, and a median significantly higher than that of the control group ($p<0.0001$). [251]

This present study reports that, in adenomas, the relationship with FC has a high degree of variability. The majority of the included studies reported that patients with adenomas had a higher FC than healthy individuals, but lower than those with colorectal cancer. However, the specificity, that would allow FC to be used to diagnose adenomas or differentiate adenomas from other organic pathologies, is absent. Size, location or number of adenomas does not appear to significantly affect FC levels. [252, 253]

However, there is a confirmed stronger relationship between FC and colorectal cancer. FC is higher in patients with CRC in comparison to both healthy patients and other degrees of neoplasia and is therefore a sensitive marker for CRC. The current globally used standard CRC screening test is FIT, which is moderately sensitive but highly specific for CRC. [254] In FC, the inverse relationship between sensitivity and specificity concedes a low specificity for CRC. This low specificity has prevented FC from becoming a useful screening tool for diagnosing CRC; however it could potentially be used as an adjunct in screening high risk populations. [255, 256]

There are also many confounding factors which elevate FC levels including use of common drugs such as proton pump inhibitors (PPIs) and NSAIDs. [257, 258] In these studies, FC was not found to be influenced by either smoking or alcohol. [255, 253] Kronborg et al. (2000) found that diverticulosis increased FC, but not more than polyps without neoplasia, however this was contradicted by Kristinsson et al when diverticulosis was not found to influence FC levels. [253, 255] These confounding factors decrease FC use as a screening tool for CRC.

In this analysis, each study's own FC values and ranges, predominantly 10mg/l or 50µg/g, were used as a reasonable cut-off point between normal and colorectal pathologies. Patients with a negative FC would be considered low-risk for CRC. Mowat et al. (2016) found that a cut-off <50µg/g was sufficient to rule out IBD, but missed 5/28 CRC's and 17/41 higher risk adenomas. [259] In contrast other studies found a higher FC level was a more optimal cut-off point for distinguishing between CRC and normal. [247] Therefore it is unclear what the appropriate cut-off value would be to determine where CRC could be safely excluded based on FC alone. However it would appear that due to the variability and low specificity of FC in CRC that this cut-off value would be too low to be of any useful clinical or financial benefit.

Overall it is widely accepted that FC alone is a poor screening test for both adenomas and CRC. However it may have a role in clinical diagnosis and staging. Particularly as an additional diagnostic tool to rationalise use of colonoscopy (a timely, expensive and invasive test), to improve risk stratification of both symptomatic and asymptomatic patients.

Many studies have shown a significant fall in FC levels post cancer resection. Kristinsson et al. 1998 found that median FC fell significantly from 75mg/l to 10.3mg/l, after resection. [249] This has again been shown by Kristinsson et al. 2001, Lehman et al. 2014, and Borza et al. 2015. [211, 246, 260] Despite the lack of evidence supporting the use of FC in screening, this is evidence to show that FC is related to intraluminal tumour burden, and hence may be relevant to clinical diagnosis, pre-operative CRC staging and cancer follow-up. Similar data was not available for adenomas post removal in this literature review, however given the low rate of adenoma detection it would be unlikely for FC levels to change significantly following removal, and therefore unlikely for any further role for FC in adenomas.

I therefore hypothesise that patients with a larger intraluminal tumour burden should have higher FC levels. In this analysis one study reported T-stage significantly ($p=0.022$) correlating with FC [246], and two further studies showed a non-significant correlation. [247, 211] However no other study showed any significant correlation between FC and stage of CRC. If you consider intraluminal tumour burden as size of tumour rather than depth i.e. T-stage, there was only one study in this review which assessed tumour size in this manner. There was no correlation found. [211] Therefore more work is required to analyse whether larger or more advanced tumours generate greater FC levels, and can therefore be used in pre-operative CRC staging.

The utilisation of FC in pre-operative staging of CRC is a novel role. The local inflammatory response also plays an important role in staging and therefore disease progression and survival in CRC. [193, 147, 194] Given that FC is a measure of colonic inflammation it may reflect the local inflammatory response, and therefore may be another area where FC can aide staging. As stated previously the current methods of assessing local inflammation rely on tissue sampling, and

assessment of local inflammation is normally post-operative and at present not part of current CRC staging. If using a simple stool sample, the presence of an elevated FC can help discriminate the patients at risk of more advanced disease. This could potentially be a quick, simple method of advancing pre-operative CRC staging.

However it is not yet clear whether FC correlates with tumour inflammation or histopathology. In UC it has been shown that disease activity, FC and histology all correlate. [224] Lehman et al 2014 carried out the first study assessing correlation of tumour and histopathological parameters of local inflammation in colorectal cancer. [246] FC did not correlate with any of the markers of local tumour inflammation (Klintrup-Mäkinen grade, lymphocytes, neutrophils, CD3, CD4, CD8, CD45, TIA-1, granzyme B and myeloperoxidase). In 1998, Kristinsson et al. found no significant correlation in colorectal cancer between FC and markers of systemic inflammation (CRP, CEA, plasma calprotectin). [249] More work needs to be performed assessing whether FC in CRC correlates with either the systemic or local inflammatory response.

The limitations of this study are the paucity and heterogeneity of data in the papers. There is variation in the patients, countries, FC assays and cut-offs and other confounding factors. In addition the use of the random effects model means more weight is given to the smaller studies, potentially increasing bias from these smaller and potentially underpowered studies. It is difficult to account for all of these factors, but this heterogeneity itself is the reason why more data and comparative reviews are required to assess whether there is an all-encompassing conclusion.

In conclusion, in the current evidence from heterogeneous studies due to the lack of specificity or sensitivity for colorectal neoplasia, FC would be a poor screening test for neoplasia, particularly adenomas. More work would clarify whether FC could be used as a diagnostic tool, in addition to FOBT/Q-fit, to rationalise use of colonoscopy in symptomatic patients.

In CRC, the lack of specificity means that FC would also be a poor cancer screening test. However the high sensitivity of FC in CRC implies a potential role for FC in the investigation of CRC and pre-operative staging of CRC. Subsequent chapters will study the relationships between FC, disease stage and measures of the local inflammatory response.

3.5 Tables and Figures

Table 3.1 Studies reporting faecal calprotectin assays in the context of colorectal neoplasia

Author	Year	Manufacturer	Test	Units	Cut-off	Standardised Cut-off
Roseth [214]	1992		EIA	µg/l		
Roseth [251]	1993		EIA	mg/l	10mg/l	50
Gilbert [261]	1996	Nycomed Pharma	ELISA	mg/l	10mg/l	50
Kristinsson [249]	1998		EIA	mg/l	10mg/l	50
Kronborg [255]	2000	Nycomed Pharma	ELISA, PhiCal	mg/l	10mg/l	50
Ton [207]	2000	Nycomed Pharma	ELISA, PhiCal	mg/l	10mg/l	50
Kristinsson [211]	2001	Nycomed Pharma	ELISA, PhiCal	mg/l	10mg/l	50
Tibble [252]	2001		ELISA	mg/l	10mg/l	50
Kristinsson [253]	2001	Nycomed Pharma	ELISA, PhiCal	mg/l	10mg/l	50
Summerton [262]	2002	Nycomed	ELISA, PhiCal	mg/l	10mg/l	50
Tibble [227]	2002		ELISA	mg/l	10mg/l	50
Costa [256]	2003	Eurospital	ELISA, Calprest	µg/g	50µg/g	50
Limburg [248]	2003	Nycomed Pharma	ELISA	µg/g	50µg/g	50
Hoff [234]	2004	Nycomed Pharma	ELISA, PhiCal	µg/g	50µg/g	50
Chung-Faye [263]	2007		ELISA	µg/g	25µg/g	25
Damms[264]	2008	Bühlmann	ELISA	µg/g	50µg/g	50
Karl [247]	2008	NovaTec	ELISA	µg/g		
Meucci [265]	2010	Eurospital	ELISA, Calprest	mg/dl	50mg/dl	
Kalimutho [266]	2011	Eurospital	ELISA, Calprest	ng/ml	45.8ng/ml	
Kok [267]	2012	Bühlmann	ELISA, EK-CAL	µg/g	50µg/g	50
Manz [268]	2012	Bühlmann	ELISA	µg/g	50µg/g	50
Parente [241]	2012	Bühlmann	ELISA	µg/g	50µg/g	50
Pavlidis [243]	2013	Bühlmann	ELISA, EK-CAL	µg/g	50µg/g	50
Khoshbaten [245]	2014	Bühlmann	ELISA	µg/g	75.8µg/g	75.8
Lehmann [246]	2014	Viollier	ELISA	µg/g	50µg/g	50
Wang [269]	2014	Labsystem	ELISA	IU/ml		
Borza [260]	2015	Sofar Farmaceutici	Cal-Detect SOFAR (Semi-quantitative)	mg/g	15mg/g	
Mowat [259]	2015	Bühlmann	ELISA, EK-CAL	µg/g	50µg/g	50
Cubiella [270]	2016	Bühlmann	ELISA, fCAL	ng/ml		
Rutka [240]	2016	Bühlmann	Quantum Blue	µg/g	128.5µg/g	128.5
Turvill [242]	2016	Bühlmann	ELISA, EK-CAL	µg/g	50µg/g	50
Widlak [271]	2016	Thermo Fisher Scientific	ELISA, EliA	µg/g	50µg/g	50
Hogberg [272]	2017	Calpro AS	ELISA, CALPRO	µg/g	100µg/g	100
Turvill [273]	2018	Bühlmann	ELISA,	µg/g	10µg/g	10

Lue [274]	2020	Thermo Fisher Scientific	EK-CAL ELISA, EliA	µg/g	50µg/g	50

Table 3.1. Studies reporting faecal calprotectin assays in the context of CR neoplasia
EIA: enzyme immunoassay, ELISA: enzyme-linked immunosorbent assay, mg/l: milligrams/litre, mg/dl: milligrams/ decilitre, µg/g: microgram/gram, ng/ml: nanogram/millilitre, IU/ml: international unit/millilitre, Standardised Cut-off: µg/g or mg/l multiplied by 5.

Table 3.2 Patient recruitment to individual trials

Author	Year	n	Recruitment		Why FC performed?	How was CRC diagnosed?	IBD excluded?
Roseth	1992	111	SP/C	Disease specific and controls	Presents new methods for extraction and assessment of FC, and assesses preliminary	Pre study	No
Roseth	1993	206	SP/C	Disease specific and controls	Assess whether FC is useful in CRC diagnosis	Pre study	Unknown - not mentioned
Gilbert	1996	18	SP/C	Disease specific and controls	Assess FC in CRC, in comparison to faecal haemoglobin	Pre study Colonoscopy and histology	Unknown – not mentioned
Kristinsson	1998	119	SP	New dx CRC	Assessment of FC in CRC	Pre study	Unknown - not
Kronborg	2000	814	SC	Screening – high risk individuals	Assess FC for detection of adenomas in high risk individuals	Colonoscopy and histology	No
Ton	2000	238	SP/C	Disease specific and controls	Comparing new and original method of FC	Not mentioned	Unknown – not
Kristinsson	2001	155	SP	Consecutive new CRC dx	Assess FC levels in pre and post-op CRC resections, and compare to tumour characteristics	Colonoscopy and histology Barium enema	Yes
Tibble	2001	233	SY/C	Consecutive referrals to colonoscopy and controls	Compare FC and FOBT in patients with CRC and polyps to assess use as biochemical marker	Colonoscopy and histology	No
Kristinsson	2001	237	SC	First degree relatives of patients with CRC	Assess whether FC is more sensitive than FOBT in detecting colorectal neoplasia	Colonoscopy and histology	Yes
Summerton	2002	134	SY	Patients with GI sign/ symptoms referred for OGD or colonoscopy	Assess FC as a method of screening for alimentary inflammation and neoplasia	Colonoscopy and histology	No
Tibble	2002	602	SY	Consecutive patients referred to GI clinic	Assess markers of inflammation to distinguish organic from nonorganic intestinal disease	Barium imaging and/or colonoscopy	Yes
Costa	2003	239	SY/C	Consecutive patients referred to clinic and controls	Assessing FC in organic and functional bowel disorders	Colonoscopy, histology Imaging	No
Limburg	2003	412	SY/SC	Referrals to colonoscopy with hx of CR neoplasia, FH CRC or IDA	Assessed FC as a screening biomarker for colorectal neoplasia	Colonoscopy and histology	Unknown – not mentioned

Hoff	2004	2321	SC	Random invitation to population for colorectal examination screening	Assessing non-invasive tests for bowel screening – comparing FC with FOBT	Colonoscopy and histology	Yes
Chung-Faye	2007	148	SY	Clinic attendance with new lower GI symptoms or known IBD	Assessing usefulness of surrogate markers of bowel inflammation	Colonoscopy and histology	No
Damms	2008	140	SY	Referrals to colonoscopy	Assess new rapid FC test, and assess FC potential for use in screening for intestinal inflammation or CRC.	Colonoscopy and histology	No
Karl	2008	551	SY/SP	Referrals to colonoscopy with GI symptoms and specific CRC recruitment	Search for novel biomarkers to improve sensitivity of CRC detection in stool samples	Colonoscopy and histology	Yes
Meucci	2010	870	SY	Outpatient referrals for colonoscopy	Evaluate the role of FC in patients referred for colonoscopy	Colonoscopy and histology	No
Kalimutho	2011	192	SY/SP	Consecutive patients attending for colonoscopy	Compare faecal based DNA integrity to FOBT and FC, for CRC and adenoma detection	Colonoscopy and histology	Yes
Kok	2012	382	SY	Patients attending with persistent lower abdominal complaints	Assess diagnostic accuracy of point of care FC and iFOBT, in suspected organic bowel disease	Colonoscopy and histology	No
Manz	2012	538	SY	Patients attending for endoscopy with abdominal discomfort	Evaluate diagnostic value of FC in patients with abdominal discomfort	Colonoscopy and histology	Unknown – not mentioned
Parente	2012	280	SY	Patients attending GI clinic with abdominal symptoms	Assessed different faecal tests as markers for advanced neoplasia	Colonoscopy and histology	Yes
Pavlidis	2013	962	SY	Patients presenting to GP with persistent GI symptoms	Assess diagnostic performance of FC in routine general practice of symptomatic patients	Colonoscopy and histology Imaging	Yes
Khoshbaten	2014	150	SP/C	Disease specific and controls	Evaluate FC as screening marker for GI malignancy	Colonoscopy and histology	Yes
Lehmann	2014	80	SP	Known CRC, admitted for treatment	Assess FC in pre and post-op CRC resections, and compare to histology	Pre study	Unknown - not mentioned
Wang	2014	40	SP/C	Known CRC and controls	Development and testing of a faecal protein biochip for the screening of CRC	Colonoscopy and histology	Yes

Borza	2015	40	SP	Known CRC (20 with T2DM and 20 without DM)	Assess FC in patients undergoing colorectal cancer surgery, comparatively in patients with and without diabetes	Pre study	Unknown - not mentioned
Mowat	2015	755	SY	Referrals with bowel symptoms to secondary care	Diagnostic accuracy of faecal haemoglobin and FC in symptomatic patients	Colonoscopy and histology	No
Cubiella	2016	1572	SY	Consecutive patients with GI symptoms referred for colonoscopy	To try and develop a CRC predictive model, for symptomatic patients	Colonoscopy and histology	No
Rutka	2016	95	SY	Referrals for colonoscopy	Compare different faecal markers in diagnosis of colorectal adenomas and cancer	Colonoscopy and histology	Unknown – not mentioned
Turvill	2016	654	SY	"2 week wait" referrals for suspected CRC	Determine diagnostic accuracy of FC in patients referred with suspected CRC	Colonoscopy and histology Imaging	No
Widlak	2016	430	SY	Referrals for urgent lower gastrointestinal investigations	Assess FC and FIT in detection of CRC and adenoma in symptomatic patients	Colonoscopy and histology Imaging	No
Hogberg	2017	373	SY	Consecutive patients receiving a FIT or FC test	Assess FC and FIT in detecting CRC, HRA and IBD in primary care	Colonoscopy and histology Imaging	No
Turvill	2018	515	SY	Patients referred for colonoscopy from '2 week wait' colorectal clinics	Diagnostic accuracy in suspected CRC, comparison with FIT	Colonoscopy and histology	No
Lue	2020	404	SY	Symptomatic patients referred for colonoscopy	Diagnostic accuracy and cost-effectiveness of combination of FOBT and FC	Colonoscopy and histology	No

Table 3.2. Patient recruitment to individual studies
CRC: colorectal cancer, dx: diagnosis, GI: gastrointestinal, OGD: oesophago-gastroduodenoscopy, FH: family history, IDA: iron deficiency anaemia, IBD: inflammatory bowel disease, GP: general practitioner, T2DM: type 2 diabetes mellitus, FIT: faecal immunochemical test, FC: faecal calprotectin, HRA: high risk adenomas SY: Symptomatic, SC: Screening, SP: Specific, C: Controls.

Table 3.3 Median faecal calprotectin levels in colorectal neoplasia

Author	Year	n					Median			
		Total	Normal (%)	Adenoma (%)	AA (%)	CRC (%)	Normal	Adenoma	AA	CRC
Roseth	1992	111	33 (29.7)	-	-	8 (7.2)	2025µg/l	-	-	40000µg/l
Roseth	1993	206	113 (54.8)	-	-	53 (27.7)	-	-	-	50mg/l
Gilbert	1996	18	4 (22.2)	-	-	14 (77.8)	5mg/l	-	-	33mg/l
Kristinsson	1998	119	-	-	-	119 (100)	5.2mg/l	-	-	50mg/l
Kronborg	2000	814	488 (60.0)	203 (24.9)	-	23 (2.8)	6.6mg/l	9.1 mg/l	-	17.6mg/l
Ton	2000	238	59 (24.8)	-	-	149 (62.6)	26µg/g	-	-	372µg/g
Kristinsson	2001	237	114 (48.1)	73 (30.8)	17 (7.2)	5 (2.1)	11.5mg/l	14 mg/l	-	18mg/l
Tibble	2001	233	96 (41.2)	29 (12.4)	-	62 (26.6)	2.3mg/l	12 mg/l	-	101mg/l
Summerton	2002	134	28 (20.9)	6 (4.5)	-	8 (6.0)	4.5mg/l	3.8 mg/l	-	53.5mg/l
Tibble	2002	602	-	-	-	7 (1.2)	-	-	-	47mg/l
Hoff	2004	2321	1518 (65.4)	592 (25.5)	195 (8.4)	16 (0.7)	21.5µg/g	-	24 µg/g	66.1µg/g
Chung-Faye	2007	148	-	-	-	7 (4.7)	15µg/g	-	-	105µg/g
Damms	2008	140	56 (40.0)	29 (20.7)	-	8 (5.7)	25.8 µg/g	66.3 µg/g	-	164 µg/g
Karl	2008	551	252 (45.7)	-	113 (20.5)	186 (33.8)	22.4µg/g	-	27.2 µg/g	420.5 µg/g/ 350.3 µg/g
Kok	2012	382	112 (29.3)	53 (13.9)	16 (4.2)	19 (5.0)	46µg/g	71 µg/g	89 µg/g	274µg/g
Manz	2012	538	314 (58.4)	50 (9.3)	-	17 (3.2)	10µg/g	101 µg/g	-	104µg/g
Khoshbaten	2014	150	50 (33.3)			50 (33.3)	19.3µg/g			19.3µg/g
Lehman	2014	80	-	-	-	80 (100)	-	-	-	205µg/g
Wang	2014	40	20	-	-	20	116IU/ml	-	-	179.1IU/ml

						(100)					
Turvill	2016	654	-	-	-	39	-	-	-	272µg/g	
						(6.0)					
Widlak	2016	430	-	42	-	24	-	-	-	145µg/g	
				(9.8)		(5.6)					
Cubiella	2016	1572	-	-	-	214	-	-	-	120ng/ml	
						(13.6)					

Table 3.3.

Median faecal calprotectin levels in colorectal neoplasia.
CRC: colorectal cancer, AA: advanced adenoma

Table 3.4 Sensitivity and specificity data for faecal calprotectin in adenomas and advanced adenomas

Author	Year	n			Cut-off	Standardised Cut-off	Sens (%)	Spec (%)	PPV (%)	NPV (%)	Comment
		Total	Adenoma (%)	AA (%)							
Adenomas											
Kronborg	2000	814	203 (24.9)	-	10 mg/l	50	43.0	-	-	-	
Tibble	2001	233	29 (12.4)	-	10mg/l	50	55.0	85.2*	45.7*	89.3*	
Kristinsson	2001	237	73 (30.8)	17 (7.2)	10 mg/l	50	56.2	47.4	40.6*	62.8*	
					15 mg/l	75	45.2	59.6	41.8*	63.0*	
					20 mg/l	100	31.5	71.1	41.1*	61.8*	
Damms	2008	140	29 (20.7)	-	50 µg/g	50	55.0	79.0	57.0	77.0	
Kalimutho	2011	192	69 (35.9)	34 (17.7)	45.8 ng/ml		28.0	25.0*	21.0*	34.0*	
Widlak	2016	430	42 (9.8)	-	50 µg/g	50	43.0	56.0	10.0*	90.0*	
Rutka	2016	95	36 (37.9)	20 (21.1)			-	-	-	-	Faecal calprotectin significantly lower in low-risk adenoma compared to CRC
Advanced adenomas											
Hoff	2004	2321	-	195 (8.4)	50 µg/g	50	26.7*	76.1*	12.5*	89.0*	
Mowat	2015	755	-	41 (5.4)	50 µg/g	50	58.5	37.8	5.3	93.8	
					200 µg/g	200	19.5	73.7	4.3	93.8	
Lue	2020	404	41 (10.1)	39 (10)	50 µg/g	50	66.6	48.8	12.2	93.2	
Parente	2012	280	-	85 (30.4)			-	-	-	-	Significant differences between faecal calprotectin in both CRC and AA, and normal and AA ($p<0.001$)
Turvill	2016	654	-	33 (5.0)			-	-	-	-	30/33 (90.9%) patients with AA had a high faecal calprotectin

Table 3.4. Sensitivity and specificity data for faecal calprotectin in adenomas and advanced adenomas
*Calculated value, AA: advanced adenoma, PPV: positive predictive value, NPV: negative predictive value, standardised Cut-off: µg/g or mg/l multiplied by 5, -: no information available/ unable to calculate based on available information

Table 3.5 Sensitivity and specificity data for faecal calprotectin in colorectal cancer

Author	Year	n		Cut-off	Standardised Cut-off	Sens (%)	Spec (%)	PPV (%)	NPV (%)	Comment
		Total	CRC (%)							
Kronborg	2000	814	23 (2.8)	10 mg/l	50	74.0	-	-	-	
Tibble	2001	233	62 (26.6)	10 mg/l	50	90.0	-	-	-	
Hoff	2004	2321	16 (0.7)	50 µg/g	50	72.7*	76.1*	4.2*	99.5*	
Damms	2008	140	8 (5.7)	50 µg/g	50	100	79.0	40.0	100	
Meucci	2010	870	34 (3.9)	50 mg/dl		85.0	58.0	6.0	99.0	
Kalimutho	2011	192	28 (14.6)	45 .8 ng/ml		72.0	75.0	43.0*	91.0*	
Parente	2012	280	47 (16.8)	50 µg/g	50	85.7	39.7	22.2	93.3	
				416 µg/g	416	43.2	88.8	44.2	88.4	
Khoshbaten	2014	150	50 (33.3)	75.8 µg/g	75.8	80.0	84.0	-	-	
Mowat	2015	755	28 (3.7)	50 µg/g	50	82.1	38.8	5.1	98.2	
				200 µg/g	200	46.4	74.9	6.9	97.2	
Rutka	2016	95	19 (20.0)	128.5 µg/g	128.5	77.8	70.0	53.8	87.5	
Turvill	2016	654	39 (6.0)	50 µg/g	50	92.7	35.2	8.7	98.6	
Widlak	2016	430	24 (5.6)	50 µg/g	50	68.0	84.0	21.0*	98.0*	Total number for this analysis is 25 CRC (including 1 HGD)
Hogberg	2017	373	8 (2.1)	20 µg/g	20	100	51.5	4.3	100	
				50 µg/g	50	87.5	72.1	6.4	99.6	
				100 µg/g	100	50.0	85.2	6.9	98.7	
Turvill	2018	515	27 (5.2)	10 µg/g	10	74.1	66.3	10.9	97.9	For a single FC
Lue	2020	404	16 (4)	50 µg/g	50	75	48.2	5.6	97.9	

Table 3.5. Sensitivity and specificity data for faecal calprotectin in colorectal cancer

*Calculated value, CRC: colorectal cancer, PPV: positive predictive value, NPV: negative predictive value

Table 3.6 Faecal calprotectin levels in different stages of colorectal cancer

Author	Year	CRC Total n	Stage				Comment
			0/I n (%) FC (Median)	II n (%) FC (Median)	III n (%) FC (Median)	IV n (%) FC (Median)	
Gilbert	1996	14		5 (36.0)	2 (14.0)	7 (50.0)	Stage had no effect on faecal calprotectin levels
Kristinsson	1998	119	25 (21.0) 50 mg/l	33 (28.0) 65 mg/l	36 (30.0) 34 mg/l	25 (21.0) 38 mg/l	No significant difference
Kristinsson	2001	155	20 (13.0) 27 mg/l	66 (43.0) 49 mg/l	45 (29.0) 42 mg/l	23 (15.0) 48 mg/l	No significant difference
Tibble	2001	62	10 (16.0) 62.5 mg/l	24 (39.0) 115 mg/l	14 (23.0) 62 mg/l	14 (23.0) 132 mg/l	No significant difference (p>0.2)
Karl	2008	85 (186)	23 (27.0) 179.2 µg/g	27 (32.0) 550.2 µg/g	12 (14.0) 542.5 µg/g	23 (27.0) 312.8 µg/g	85/186 CRC had stage specified
Kalimutho	2011	28	7 4/7 +ve FC	5 3/5+ve FC	3 3/3 +ve FC		18 CRC either did not have FC or stage data
Lehman	2014	80					Patients with T3/4 disease had significantly higher FC than T1/2 (p=0.022)

Table 3.6. Faecal calprotectin levels in different stages of colorectal cancer
CRC: colorectal cancer, FC: faecal calprotectin

Table 3.7 Faecal calprotectin levels in different locations of colorectal cancer

Author	Year	CRC Location					Comment
		CRC Total n	Colon n (%) FC (Median)	Rectum n (%) FC (Median)	Left n (%) FC (Median)	Right n (%) FC (Median)	
Gilbert	1996	14	13 (93.0)	1 (7.0)	79.3 mg/l (mean)	55.1 mg/l (mean)	No significant difference (p=0.4)
Kristinsson	1998	119	81 (68.0) 50.0 mg/l	38 (32.0) 54.5 mg/l	73 (61.0) 77.4 mg/l	46 (39.0) 61.6 mg/l	No significant difference
Kristinsson	2001	155	106 (68.0) 41.5 mg/l	49 (32.0) 53 mg/l			No significant difference
Tibble	2001	62	31 (50.0)	31 (50.0)			No significant difference (p>0.5)
Limburg	2003						Patients with proximal colonic neoplasms had a higher median FC than distal (Proximal 53.8µg/g, distal 23.0 µg/g P=0.001)
Lehman	2014	80					No significant difference
Widlak	2016	24			143 µg/g	175 µg/g	No significant difference (p=0.7068)

Table 3.7. Faecal calprotectin levels in different locations of colorectal cancer
CRC: colorectal cancer, FC: faecal calprotectin

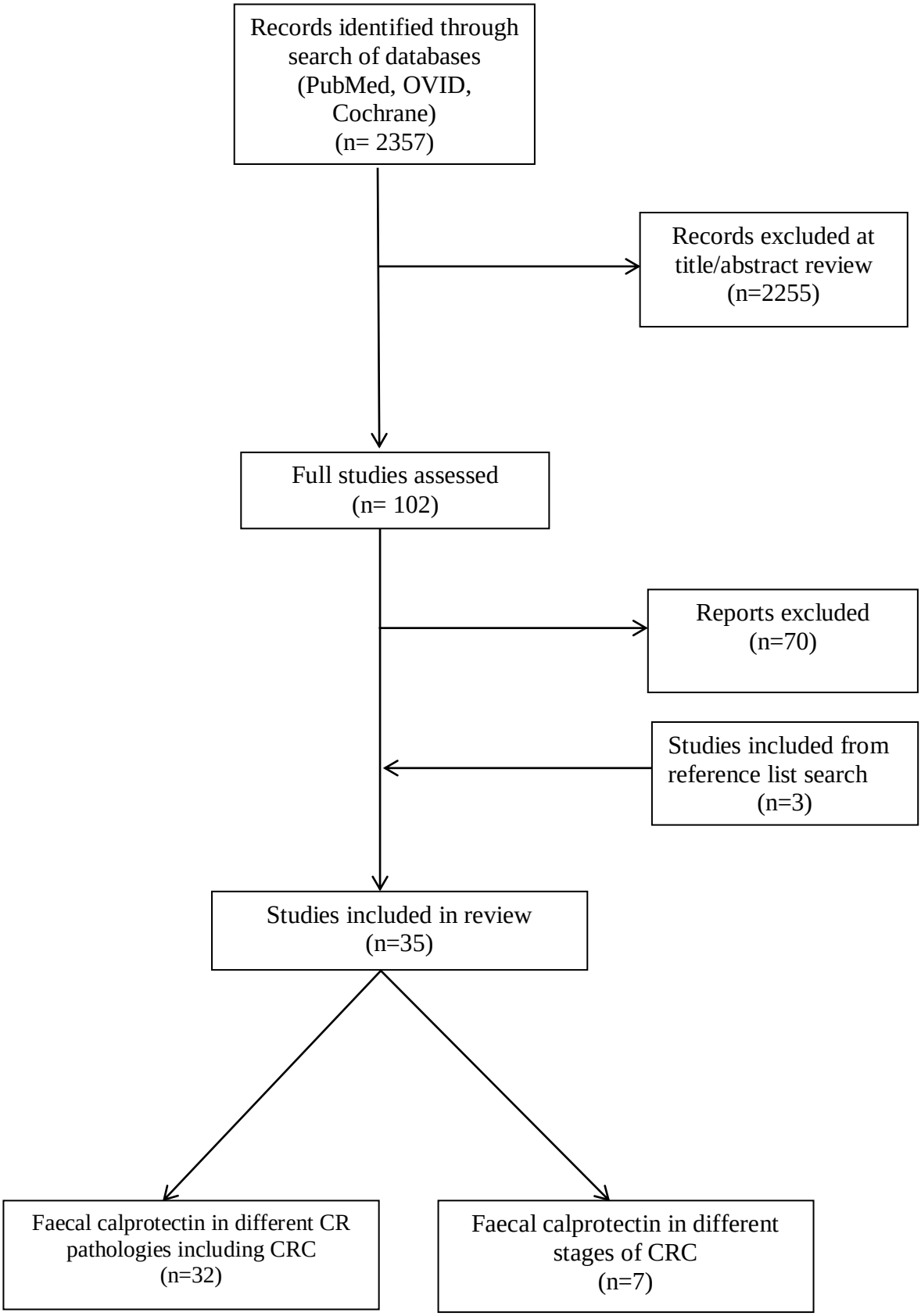

Figure 3.1. PRISMA flow chart of study selection process

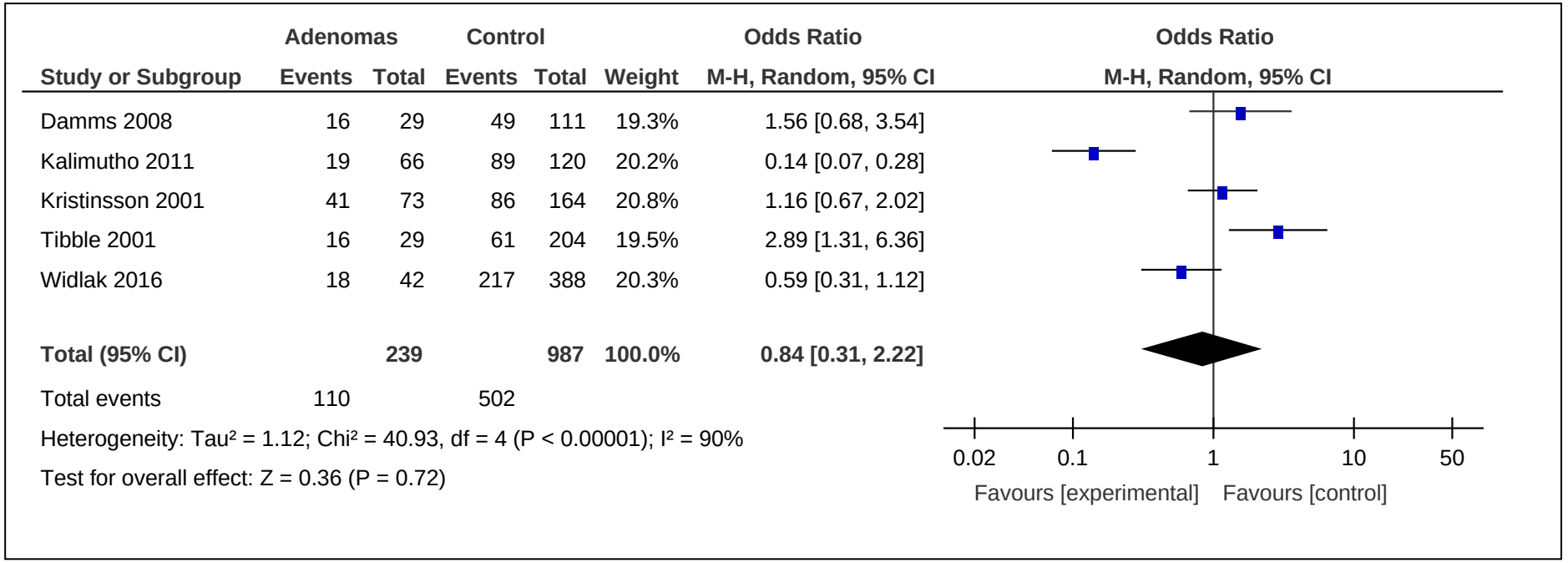

Figure 3.2a. Forest plot - faecal calprotectin in adenoma

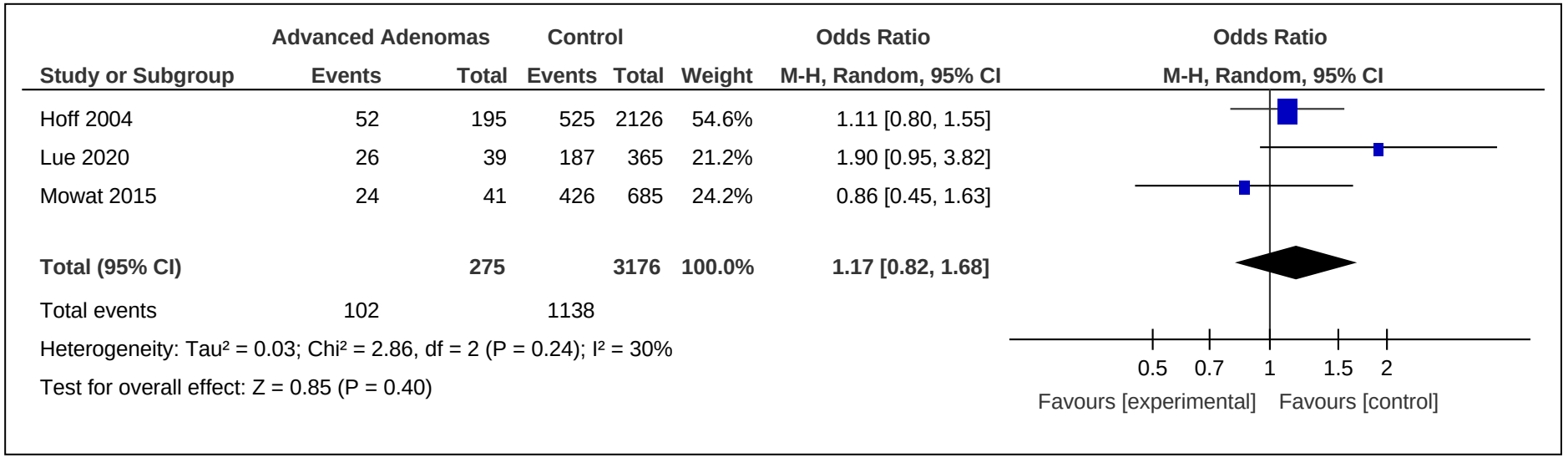

Figure 3.2b. Forest plot - faecal calprotectin in advanced adenoma

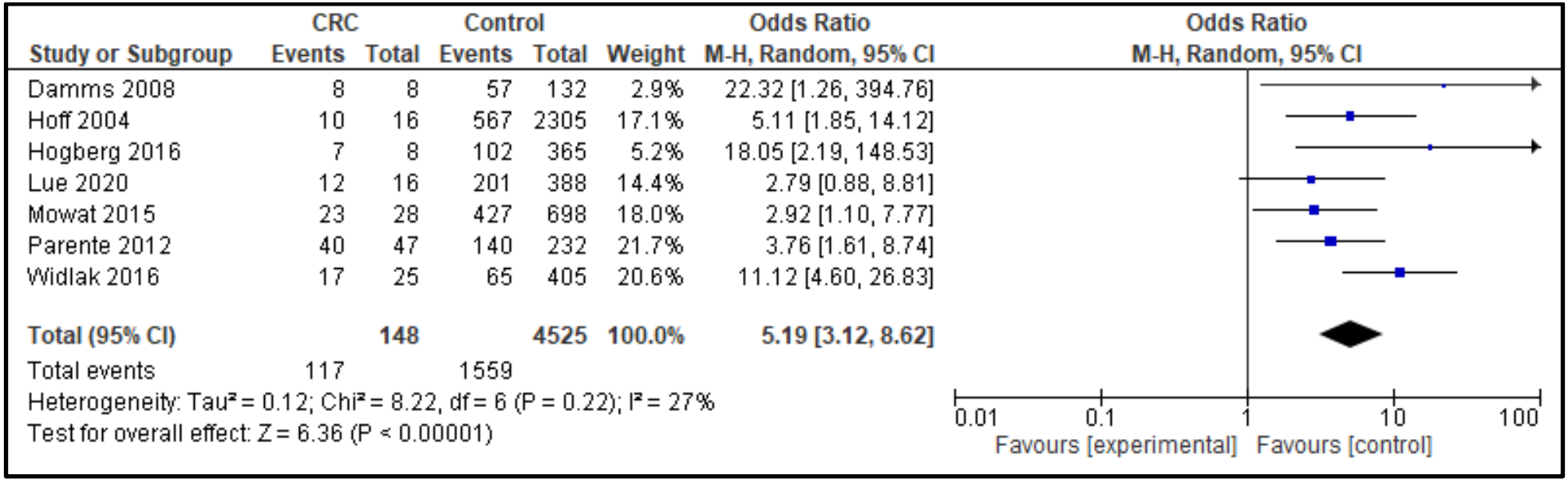

Figure 3.3. Forest plot - faecal calprotectin in cancer

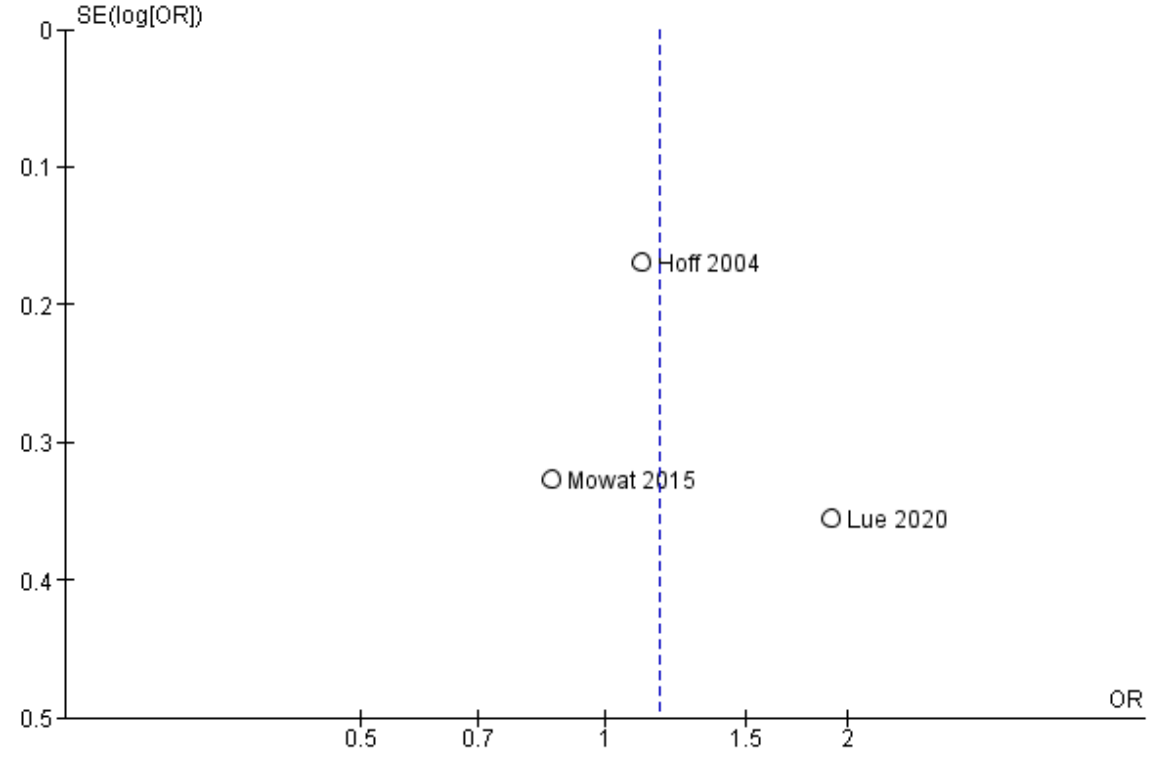

Figure 3.4a. Funnel plot of comparison: Faecal Calprotectin in Advanced Adenomas

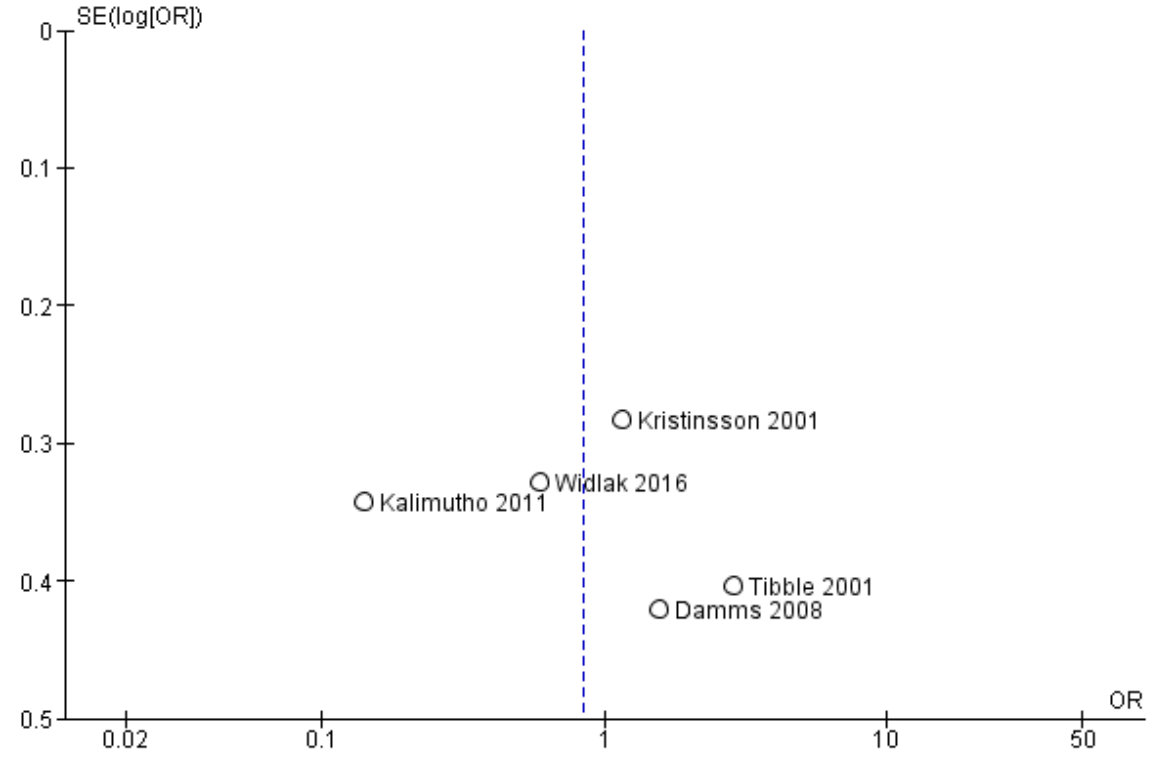

Figure 3.4b. Funnel plot of comparison: Faecal Calprotectin and Adenomas

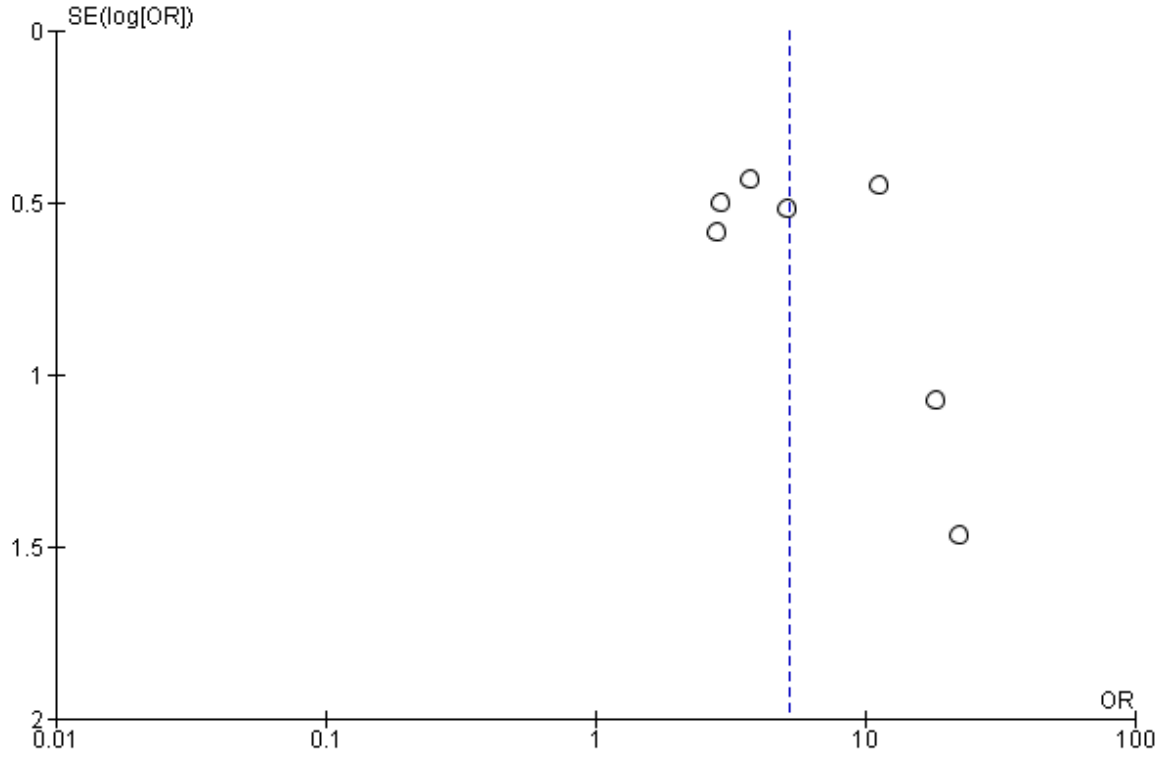

Figure 3.4c. Funnel plot of comparison: Faecal Calprotectin and CRC

4.0 The role of faecal calprotectin in the identification of colorectal neoplasia in patients attending for screening colonoscopy

4.1 Introduction

The Scottish Bowel Screening Programme was introduced across Scotland in 2007 with the aim to increase the number of cancers diagnosed at an early stage, where the disease is more readily treatable. Based on trials, screening was anticipated to reduce CRC mortality by as much as 15%. [275] Up to half a million people participate in bowel screening in Scotland each year. [276]

The current biennial screening programme in Scotland invites patients aged 50-74yrs to participate in an initial stool test (previously gFOBT and now replaced by FIT). If this returns a positive result colonoscopy is scheduled. Locally, current uptake of bowel screening is only 55% and the current rate of CRC diagnosis in screening colonoscopies is 6-8%. [277] Colonoscopy is an invasive, expensive test and patients and health care professionals agree strategies to improve current screening models are required. Improving the sensitivity and specificity for CRC diagnosis with either a novel test or optimisation of the current testing strategy is extremely attractive, representing a 'holy grail' in bowel cancer research.

Current stool testing evaluates for blood lost from the surface of neoplastic lesions. However, other cancer associated phenomena may be measured in stool samples. One example is the presence of inflammation which is recognised as the seventh hallmark of cancer. [173] Specifically the local immune response is a key determinant of carcinogenesis and malignant progression in CRC in addition to conferring prognostic value. [193, 147, 194]

FC is an established marker of inflammation used predominantly in IBD. [206, 223] However the association between faecal calprotectin, and colorectal neoplasia is less well defined, particularly within the screening age cohort (50-74yrs). Evidence suggests that FC has a high sensitivity in CRC, despite a lack of specificity [272], with a high negative predictive value (NPV) in a symptomatic population. [242] In a screening age population, one previous study by Hoff et al 2014 reported CRC presence was associated with significantly higher calprotectin values [234], but this was limited by the selective inclusion process meaning that the faecal calprotectin data is not representative of a full screening population. I hypothesise that FC may have a role in providing additional discriminatory value for the presence/ absence of colorectal neoplasia within a population already preselected for screening colonoscopy based on FOBT/FIT positivity. If this were to be demonstrated, it may be possible to refine the stool testing to more accurately direct resources such as colonoscopy.

The aim of this prospective study was to determine the relationship between FC measurements and the presence of colorectal neoplasia in a cohort of patients presenting for colonoscopy via the SBSP.

4.2 Methods

4.2.1 Patients

The present study's results were obtained as part of a larger prospective 'Investigation of the local and systemic inflammatory response and of dietary habits in those attending for investigation via the National Health Service Colorectal Cancer Screening Programme'. As part of this study, faecal calprotectin measurements were obtained.

All patients attending for bowel cancer screening colonoscopy, within the SBSP, between February 2016 and July 2017, at four NHS Greater Glasgow and Clyde Hospitals (Glasgow Royal Infirmary, Gartnavel General Hospital, New Stobhill Hospital and New Victoria Hospital) were invited to participate in this prospective study.

The analysis of asymptomatic FOBT positive patients from a bowel screening programme, allows for a more novel approach compared to other studies which have predominantly focused on symptomatic patients. This will assess FC and whether it will provide additional discriminatory value for colorectal neoplasia within a population already preselected for screening colonoscopy.

4.2.2 Methods

The parent study 'Investigation of the local and systemic inflammatory response and of dietary habits in those attending for investigation via the National Health Service Colorectal Cancer Screening Programme' study proposal was already in place. I set up and managed the study going forward including putting this study through ethics approval, planning and organising the logistics of the study, day-to-day running and management, data collection and analysis.

Using the bowel screening colonoscopy waiting lists, patients undergoing screening colonoscopy following a positive FOBT test were identified. Approximately 2-3 weeks prior to attendance for colonoscopy, patients were posted an information pack about the study. This pack contained a cover letter, information leaflet on the study, consent form, food frequency questionnaire (FFQ) and a calprotectin kit for collection of their faecal sample.

Patients had from the time they received this pack until their arrival at the endoscopy unit to consider their decision to participate in this study. The paperwork could be completed at home, or while in the endoscopy unit. The consent form was countersigned by the member of nursing staff looking after the patient, after the opportunity to ask questions was given. The consent form, along with the completed FFQ was collected from the endoscopy units by research staff.

Consenting patients were instructed to provide a stool sample for calprotectin. This was to be taken as close as possible to their colonoscopy date, but prior to commencement of bowel preparation. The patient then returned the sample on the day of their colonoscopy. Faecal calprotectin was

analysed by the NHS biochemistry laboratory, at Glasgow Royal infirmary using standard clinical grade assays (Bühlmann fCAL® ELISA).

Patient demographics and colonoscopy results were recorded in a prospectively maintained database on an encrypted hard drive. Colonoscopy, blood and faecal calprotectin results were obtained from the patient's digital record. Using this system additional data including smoking status, drug use and BMI (if not measured on the day) were obtained.

The final cohort of patients were sub-divided based on the final pathology results to allow comparison of neoplasia and inflammatory conditions. Neoplasia is subdivided into non-advanced adenoma (adenoma without advanced features), advanced adenoma (adenoma with one advanced feature of high grade dysplasia, ≥1cm at pathology, villous), advanced neoplasia (grouping of all advanced neoplasia and malignancy patients) and cancer. The categories are fully listed and described in Table 4.1. Patients were excluded if there was incomplete data regarding the colonoscopy or resultant pathology, or if they had previously undergone a colorectal resection.

4.2.3 Objectives

The primary study objective was to analyse the relationship between FC and CRC, in an FOBT positive bowel screening cohort of patients. Secondary objectives included evaluation of the relationship between FC and lesser forms of neoplasia including adenomas and advanced adenomas.

4.2.4 Statistical Analysis

Categorical data regarding patient characteristics were compared using the Chi square test and Chi square test for linear association where appropriate. Local studies suggest that, in adult patients, FC values <200µg/g are rarely associated with IBD or other significant luminal pathology. [220, 221] Local guidelines and reference range therefore deems <50µg/g as normal, but only >200µg/g is deemed clinically significant. [222] Faecal calprotectin was therefore divided into 3 categories for analysis based upon these local thresholds resulting in 3 groups: <50µg/g, 50-200µg/g and >200 µg/g. In addition, faecal calprotectin results were expressed as medians, and analysed using Kruskal-Wallis. Binary logistic regression was used to examine the relationship between patient factors and faecal calprotectin thresholds, and calculate an OR and 95% CI. Factors that on univariate analysis had a p value <0.10 were taken into a multivariate model using a backward conditional approach to identify independently significant factors. Sensitivity and specificity was calculated using normal as the control for all categories. A p value of <0.05 was considered significant for all analyses. Statistical analysis was performed using SPSS version 25.0 for Windows (IBM Corporation, Armonk, NY).

4.2.5 Ethics

This study was approved by the NHS Greater Glasgow and Clyde Research and Development department, and the Research Ethics Service (REC number: 15/WA/0053).

4.3 Results

Two thousand and thirty three patients were invited to participate in the study. Four hundred and fifty six patients consented to participate and returned the relevant paperwork; twelve patients were excluded therefore four hundred and forty four patients were included in the final analysis. Figure 4.1 displays the three hundred and fifty two of these patients returned a faecal calprotectin stool sample making up the final study number.

4.3.1 Patient Characteristics

Table 4.2 describes the baseline patient demographics from study participants. The majority were female (n=186, 53%) and over the age of 60 years (n=221, 63%). A large proportion (n=102, 29%) were classed in the lowest (most deprived) Scottish Index of Multiple Deprivation (SIMD) quintile. The majority had a BMI <30 (n=202, 64%) and 164 (51%) had ever smoked. 146 (42%) of patients were currently on a PPI. 210 (60%) patients had an elevated FC ≥50µg/g and 90 (26%) of patients had a FC more than 200 µg/g.

4.3.2 Patient characteristics and faecal calprotectin

Table 4.3 details the relationship between patient characteristics and faecal calprotectin value. In summary higher FC values were associated with higher BMI (P<0.05), NSAID use (p=0.006) and PPI use (p<0.001). Age, sex, SIMD quintile, smoking, aspirin, statin, metformin and ACE inhibitor use were not associated with FC.

4.3.3 Pathology and faecal calprotectin

Table 4.4. details the relationship between colonic pathology and faecal calprotectin. The colonic pathology categories described in Table 4.1. are analysed for their associations with FC.

A higher FC was associated with inflammatory (p<0.05) and CRC (p<0.05) pathology. 60% (n=210) patients with a normal colonoscopy had an elevated FC (>50µg/g), with a median FC of 81.0µg/g. The majority (n=14, 93%) of inflammatory patients had an elevated FC (>50µg/g), with a median FC 166.0µg/g (p<0.05). The majority (n=13, 93%) of CRC patients had an elevated FC (>50µg/g), with a median FC 138.5 µg/g (p<0.05). There was no significant relationship observed between FC and non-cancer neoplasia. The relationship between FC and inflammatory and CRC remained even after excluding patients prescribed PPI and NSAIDs.

Table's 4.5a/b. show binary logistic regression of both the colonic pathology and patient characteristics associated with faecal calprotectin. On univariate analysis aspirin use [odds ratio (OR) 1.97, 95% confidence interval (CI) 0.95-1876, p<0.05], NSAID use (OR 2.72, 95% CI 1.06-3.66, p=0.06), PPI use (OR 2.74, 95% CI 1.74-4.33, p <0.001), inflammatory pathology (OR 10.07, 95% CI 1.31-77.48, p<0.05) and CRC (OR 9.31, 95% CI 1.2-71.95, p<0.05) were positively associated with FC >50µg/g. Non-advanced adenoma (OR 0.57, 95% CI 0.34-0.96, p<0.05) was

inversely associated with FC >50µg/g. On multivariate analysis aspirin use (OR 2.21, 95% CI 1.15-4.24, p<0.05), NSAID use (OR 2.41, 95% CI 1.14-5.12, p<0.05), PPI use (OR 2.57, 95% CI 1.58-4.19, p<0.001), inflammatory pathology (OR 13.13, 95% CI 1.67-102.93, p<0.05) and CRC (OR 16.62, 95% CI 2.12-130.56, p<0.05) remained independently associated with FC >50µg/g.

On repeating the binary logistic regression analysis with a FC threshold of 200 µg/g NSAID use (OR 1.8, 95% CI0.95-3.39, p=0.071) and PPI use (OR 2.95, 95% CI 1.80-4.84, p <0.001) were positively associated with FC. BMI was also associated with FC (OR 1.72, 95% CI 1.03-2.87, p=0.037). On multivariate analysis only PPI use (OR 3.16, 95% CI 1.87-5.31, p <0.001) was independently associated with a FC >200µg/g.

The sensitivity specificity data is displayed in Table 4.6. FC had a high sensitivity for inflammatory pathology (93.3%, 95%CI 68.1-99.8%), with a NPV 99.3% (95%CI 95.5-99.9). FC sensitivity increased sequentially as neoplasm progressed from non-advanced to malignant neoplasia (48.6% non-advanced adenoma vs. 92.9% CRC). Malignancy has a high sensitivity (92.9% 95% CI 66.1-99.8), at a cut off of 50µg/g. For malignancy this cut-off has a negative predictive value 99.3% (95% 95.5-99.9). However there was low specificity, which improved when FC cut off was raised to 200µg/g.

4.4 Discussion

This prospective study aimed to characterise the relationship between FC and colorectal neoplasia, in a population undergoing colonoscopy following a positive bowel screening test. Whereas prior studies have predominantly focused on symptomatic patients, the present study is novel in its inclusion of asymptomatic FOBT positive patients from a bowel screening programme.

This study demonstrated that a high FC is associated with CRC, with 93% (n=13/14) of the CRCs detected in this study having an elevated FC (>50µg/g). However, although sensitive for the detection of CRC, FC failed to show sufficient sensitivity nor specificity for the detection of non-cancer colorectal neoplasia in a FOBT positive bowel screening cohort. Therefore, FC alone would not be sufficient on its own as a screening tool for CRC neoplasia.

Colorectal neoplasia covers a wide range of pathology from small adenomatous polyps through to colorectal cancers. Previous studies have suggested that patients with adenomas had a higher FC than healthy individuals, but lower than those with colorectal cancer. [241, 252, 278] In this study patients with CRC had a higher FC, with a trend towards a stepwise increase in median FC from a median FC of 48.5µg/g in non-advanced adenoma, 54.5µg/g in advanced adenoma, 68.0µg/g in advanced neoplasia and 138.5µg/g in CRC. Furthermore, this association was evident after controlling for confounding factors. However, with the exception of CRC, other forms of neoplasia did not significantly raise the median FC above those with a normal colonoscopy (median FC 81.0µg/g). Other studies have reported the lowest median FC in normal patients, rising to the highest in CRC patients, but in non FOBT/FIT screened cohorts. [255, 253, 252, 234, 264, 247, 267, 268] In our study the median FC was lower in non-cancer neoplasia than in patients with normal screening colonoscopy. It is unclear why this is the case in this screening cohort but it does suggest that as a population screening test, FC (in addition to FIT/FOBT) is not useful to distinguish non-malignant neoplasia from other colorectal pathology or even patients with no pathology at all.

In this study FC (cut-off of 50µg/g) has a low sensitivity for non-cancer neoplasia. Non-advanced adenomas have a sensitivity of 48.6% and NPV 73.9%. This is the similar to other studies (43-56.2% sensitivity and NPV 77%). [264, 271, 253, 252, 255] Advanced adenomas have a sensitivity of 51.4% and NPV 76.1% in our study, lower than other studies (sensitivity 58.5-66.6% and NPV 93.2-93.8%).[259, 274] Mowat et al, analysed the geographically most similar cohort to ours, yet reported higher sensitivity and NPV. Most of the studies assessed symptomatic cohorts but Kronberg et al, and Kristinsson et al both analysed high risk screening cohorts. The sensitivity for adenomas in our study was midway between that reported in these studies (43 and 56.2% respectively). [253, 255] Taken together, these results indicate that FC does not reliably discriminate between patients with or without premalignant neoplasia.

However, there is a stronger relationship observed between FC and CRC. CRC has a higher median FC (138.5µg/g) than all other pathology, with the exception of inflammatory pathology. FC, at a cut-off of 50µg/g, has a high sensitivity (92.8%) and NPV (99.3%) for CRC. This NPV is similar to what is reported elsewhere in the literature but our reported sensitivity is higher than that reported in t some of the literature, with Lue *et al* reporting 75%, Mowat *et al* 82.1% and Hogberg *et al* 87.5%, but similar to others including another UK based study Turvil *et al* 92.7%. [242, 259, 274, 272] Even with the high sensitivity and NPV, within the current FOBT positive population, one patient out of 14 would have had a CRC missed if access to colonoscopy was determined by additional FC testing. All studies, including ours report low specificity and PPV. This reflects the high accuracy of FC at detecting pathology, but not CRC alone.

FC is a marker of gastrointestinal tract inflammation, but there are many other confounding factors that can elevate FC. This study indicated both PPI and NSAID use as factors which can elevate FC, in addition to BMI. The causative mechanism by which PPI elevates FC levels is uncertain, but it is thought to be multi-factorial. Potentially mechanisms include bacterial overgrowth due to acid inhibition and the anti-oxidative properties of PPI, an association with upper GI inflammation has been hypothesised but this has not been shown to correlate. [257, 279] NSAID related FC elevation is due to induced enteropathy. [258] Other factors including age, sex, and smoking status did not appear to elevate FC. These results are in line with previously reported literature. [257, 258]

Some studies have questioned whether other benign colorectal conditions such as diverticulosis would be a confounding factor. [255] While this wasn't the aim of this study, there was no evidence that in our cohort factors out with those mentioned above or pathologies of colorectal cancer or inflammation elevate FC. Many factors may contribute to the lack of association with diverticulosis including the incidence of reporting diverticulosis, and the varying degrees of severity of diverticulosis.

There are a number of limitations with this study. The patients in this study are selected from a FOBT positive bowel screening population. Patients who participate in cancer screening are not representative of the population as a whole. It is known that various patient factors determine who agrees to participate in bowel screening. In the UK, deprivation is associated with both lower uptake of initial bowel screening as well as lower numbers proceeding to colonoscopy. Younger, male patients are also less likely to participate. [280] Therefore this is a different cohort of patients from the symptomatic patients more commonly included in FC studies. Another limitation is the timing of analysis of FC. Patients were instructed to provide their stool sample, as close as possible to their colonoscopy appointment, but prior to the commencement of bowel preparation. This means that patients obtained their sample at least 24 hours prior to returning it for analysis (due to the timing of bowel preparation). This reflects working clinical practice; patients return stool samples for analysis and the clinician will be unaware of how long elapsed from obtaining to

processing the sample. Stool samples for FC can be stored for up to 3 days, without refrigeration. [216, 217] However it is preferable to analyse the sample as soon as possible, and any delays or variations in time of analysis could affect the FC results. Not all patients who consented to participate in the study had valid faecal calprotectin samples. The patients in this cohort are bowel screening patients, and therefore have previously returned faecal samples for FOBT, reducing the likelihood of the patients having difficulty collecting the faecal sample. There were a small number of returned samples which were discarded by the lab, due to unacceptable sample labelling. The specific reasons were not collected as part of this study, however it would be useful information to learn in the future, to ensure there are not modifiable factors. The sample size was calculated based on power calculations for the original study, however the low specificity reported in our study would not support a larger powered study. The small number of CRCs (n=14) in this study limited more extensive description and analysis of FC in CRC. TNM staging was as follows: stage I (n=6), stage II (n=1), stage III (n=6), stage IV (n=1). However this is potentially an area of interest for further work.

In current practice FC is primarily used in diagnosis and monitoring of IBD. There may be additional uses for FC out with the remit of IBD. In healthcare there are limited resources to investigate and manage patients. Historically patients with clinically suspicious symptoms were investigated directly with colonoscopy. However colonoscopy is a timely, expensive and invasive test. While performing FC is not without cost, it is less expensive and less invasive than colonoscopy. For these reasons quantitative faecal immunochemical testing (qFIT) is now used to triage symptomatic patients (as well as in bowel screening) that are referred for colonoscopy. [6] There is evidence that symptomatic patients with a low qFIT (<10µg/g) are unlikely to be diagnosed with CRC, conversely those with a qFIT >10 µg/g do require further investigation. [281, 259] It has been proposed that higher thresholds could help guide referral and further investigation. While a higher threshold applicable to symptomatic patients does stratify risk for potential presence of CRC, it also lacks specificity and so a much lower threshold remains applicable in the screening population. [281] As discussed previously all of the patients in this study had a positive FOBT. Lue et al have shown that a combination of FOBT and FC has better diagnostic accuracy than each test alone, and it may be that the combined use is the reason for the higher sensitivity for CRC in our cohort. [274]

While FC cannot be recommended as an isolated screening tool for CRC neoplasia, it remains to be determined whether it could be implemented alongside other screening and diagnostic modalities to improve risk stratification of both symptomatic and asymptomatic patients. This would require further investigation in the context of a prospective study. [281, 282] In this study within a screened population, I conclude that the benefit from adding FC as a discriminatory test within a FOBT positive population is modest. Based on a threshold of 50µg/g, 93% of CRCs, and 50% of

adenomas would be identified. However, 60% of FOBT positive patients with a subsequent normal colonoscopy also had an elevated FC, and therefore would have merited colonoscopic assessment.

In a FOBT positive screening population, FC was strongly associated with CRC (sensitivity 92.8% for CRC, at 50µg/g) but lacked specificity. FC also failed to show sufficient sensitivity and specificity for the detection of non-cancer neoplasia. Based on these results I cannot recommend routine use of FC in a bowel screening population to detect cancer, but it is apparent that with further optimisation, faecal assessments including quantification of haemoglobin and inflammation could form part of a risk assessment tool aimed at refining selection of patients for colonoscopy in both symptomatic and screening populations.

4.5 Tables and Figures

Table 4.1 Patient Categories	
Normal	No pathology
Other	Non-inflammatory, non-neoplastic pathology i.e. diverticulosis, haemorrhoids
Inflammatory	Active inflammatory conditions i.e. colitis, diverticulitis
Neoplastic	
Non-advanced adenoma	Adenoma without advanced features
Advanced adenoma	Adenoma with one advanced feature of high grade dysplasia, ≥ 1cm at pathology, villous
Advanced neoplasia	Advanced adenoma and/ or malignancy
Cancer	Malignancy only

Table 4.1. Patient Categories

Table 4.2 Clinical characteristics	
Characteristic	All patients n (%)
Total number of patients	352
Age (years)	
50-59	131 (37.2)
60-69	166 (47.2)
70+	55 (15.6)
Sex	
Female	186 (52.8)
Male	166 (47.2)
SIMD Quintile	
1	102 (29.0)
2	61 (17.3)
3	54 (15.3)
4	56 (15.9)
5	79 (22.4)
BMI (317)[a]	
<30	202 (63.7)
30+	115 (36.3)
Smoking (336)[a]	
Never	164 (48.8)
Ever	172 (51.2)
Drugs (number of patients on)	
Aspirin	58 (16.5)
NSAIDs	50 (14.2)
PPI	146 (41.5)
Statin	142 (40.3)
Metformin	23 (6.5)
ACE	70 (19.9)
Faecal Calprotectin	
<50	142 (40.3)
50-200	120 (34.1)
>200	90 (25.6)
Bowel screening investigation outcome	
Normal	116 (33.0)
Other	65 (18.5)
Inflammatory	15 (4.3)
Non-advanced adenoma	72 (20.5)
Advanced adenoma	70 (19.9)
CRC	14 (4.0)
Bowel screening investigation outcome (185) *Excluding inflammatory, PPI and NSAID*	
Normal	51 (27.6)
Other	32 (17.3)
Non-advanced adenoma	41 (22.2)
Advanced adenoma	49 (26.5)
CRC	12 (6.5)

Table 4.2. Clinical Characteristics
SIMD: Scottish Index of Multiple Deprivation BMI: body mass index NSAIDS: non-steroidal anti-inflammatory drugs, PPI: proton pump inhibitors, CRC: colorectal cancer
a Number of patients when incomplete data available

Table 4.3 Relationship between patient characteristics and faecal calprotectin

		Median FC	P-value	FC <50 n (%)	FC 50-200 n (%)	FC >200 n (%)	P-value*
All patients		77.5 (29.0-210.8)	-	142 (40)	120 (34)	90 (20)	-
Age (years)							
<60		59.0 (29.0-220.0)	0.219	59 (45.0)	37 (28.2)	35 (26.7)	0.156
60-69		78.5 (29.0-169.3)		65 (39.2)	66 (39.8)	35 (21.1)	
70+		92.0 (29.0-285.0)		18 (32.7)	17 (30.9)	20 (36.4)	
Sex							
Female		78.5 (29.0-236.5)	0.824	79 (42.5)	58 (31.2)	49 (26.3)	0.736
Male		75.0 (29.0-199.3)		63 (38.0)	62 (37.3)	41 (24.7)	
SIMD Quintile							
1		96.5 (29.0-259.3)	0.113	35 (34.3)	35 (34.3)	32 (31.4)	0.172
2		71.0 (29.0-195.5)		26 (42.6)	21 (34.4)	14 (23.0)	
3		54.5 (29.0-161.8)		24 (44.4)	20 (37.0)	10 (18.5)	
4		90.0 (29.0-237.3)		21 (37.5)	18 (32.1)	17 (30.4)	
5		58.0 (29.0-169.0)		36 (45.6)	26 (32.9)	17 (21.5)	
BMI (316)[a]							
<30		71.0 (29.0-182.3)	0.032	85 (42.1)	72 (35.6)	45 (22.3)	0.034
30+		96.0 (29.0-258.0)		38 (33.0)	39 (33.9)	38 (33.0)	
Smoking (336)[a]							
Never		69.5 (29.0-219.5)	0.909	65 (39.6)	55 (33.5)	44 (26.8)	0.690
Ever		77.5 (29.0-186.0)		70 (40.7)	60 (34.9)	42 (24.4)	
Drugs							
Aspirin	N	69.5 (29.0-208.3)	0.190	126 (42.9)	93 (31.6)	75 (25.5)	0.174
	Y	89.5 (42.8-218.5)		16 (27.6)	27 (46.6)	15 (25.9)	
NSAIDs	N	68.5 (29.0-188.8)	0.029	131 (43.4)	99 (32.8)	72 (23.8)	0.006
	Y	114.5 (50.8-244.3)		11 (22.0)	21 (42.0)	18 (36.0)	
PPI	N	50.0 (29.0-147.5)	<0.001	103 (50.0)	68 (33.0)	35 (17.0)	<0.001
	Y	115.5 (47.8-260.0)		39 (26.7)	52 (35.6)	55 (37.7)	
Statin	N	68.5 (29.0-203.3)	0.614	90 (42.9)	67 (31.9)	53 (25.2)	0.417
	Y	83.5 (29.0-213.5)		52 (36.6)	53 (37.3)	37 (26.1)	
Metformin	N	73.0 (29.0-199.5)	0.388	133 (40.4)	114 (34.7)	82 (24.9)	0.518
	Y	97.0 (37.0-256.0)		9 (39.1)	6 (26.1)	8 (34.8)	
ACE	N	78.5 (29.0-214.3)	0.331	112 (39.7)	96 (34.0)	74 (26.2)	0.541
	Y	67.0 (29.0-172.3)		30 (42.9)	24 (34.3)	16 (22.9)	

Table 4.3. Relationship between patient characteristics and faecal calprotectin
FC: faecal calprotectin SIMD: Scottish index of multiple deprivation BMI: body mass index NSAIDs: non-steroidal anti-inflammatory drugs, PPI: proton pump inhibitors, CRP: C - reactive protein
a Number of patients when incomplete data available ∗ chi squared linear by linear

Table 4.4 Relationship between colonic pathology and faecal calprotectin							
Categories	n	Median FC	P-value	FC <50 n (%)	FC 50-200 n (%)	FC >200 n (%)	P-value*
All Patients	352	77.5 (29.0-210.8)	-	142 (40.3)	120 (34.1)	90 (25.6)	-
Normal	116	81.0 (29.0-235.0)	0.614	45 (38.8)	39 (33.6)	32 (27.6)	0.557
Other	65	90.0 (30.5-251.5)	0.232	24 (36.9)	22 (33.8)	19 (29.2)	0.429
Inflammatory	15	166.0 (68.0-404.0)	0.034	1 (6.7)	8 (53.3)	6 (40.0)	0.025
Non-advanced adenoma	72	48.5 (29.0-177.0)	0.032	37 (51.4)	20 (27.8)	15 (20.8)	0.060
Advanced adenoma	70	54.5 (29.0-150.5)	0.084	34 (48.6)	23 (32.9)	13 (18.6)	0.204
Advanced neoplasia	84	68.0 (29.0-169.3)	0.535	35 (41.7)	31 (36.9)	18 (21.4)	0.473
CRC	14	138.5 (88.8-276.3)	0.030	1 (7.1)	8 (57.1)	5 (35.7)	0.038

Table 4.4. Relationship between colonic pathology and faecal calprotectin
∗chi squared linear by linear

Table 4.5a Binary logistic regression of patient characteristics and colonic pathology associated with faecal calprotectin (cut-off 50µg/g)

Characteristic	Univariate analysis OR (95% CI)	P-value	Multivariate analysis OR (95% CI)	P-value
Patient characteristic				
Age	1.29 (0.95-1.76)	0.106	-	-
Sex	1.21 (0.79-1.85)	0.388	-	-
SIMD	0.92 (0.79-1.05)	0.215	-	-
BMI	1.47 (0.91-2.38)	0.113	-	-
Smoking	0.96 (0.62-1.48)	0.842	-	-
Aspirin	1.97 (1.06-3.66)	0.032	2.21 (1.15-4.24)	0.017
NSAIDs	2.72 (1.34-5.51)	0.06	2.41 (1.14-5.12)	0.021
PPI	2.74 (1.74-4.33)	<0.001	2.57 (1.58-4.19)	<0.001
Statin	1.3 (0.84-2.01)	0.242	-	-
Metformin	1.06 (0.44-2.51)	0.903	-	-
ACE Inhibitor	0.88 (0.52-1.49)	0.632	-	-
Colonic Pathology				
Normal	1.10 (0.70-1.74)	0.678	-	-
Other	1.19 (0.68-2.08)	0.534	-	-
Inflammatory	10.07 (1.31-77.48)	0.027	13.13 (1.67-102.93)	0.014
Non-advanced adenoma	0.57 (0.34-0.96)	0.033	0.69 (0.36-1.20)	0.186
Advanced adenoma	0.66 (0.39-1.11)	0.118	-	-
Advanced neoplasia	0.93 (0.57-1.53)	0.777	-	-
CRC	9.31 (1.20-71.95)	0.033	16.62 (2.12-130.56)	0.008

Table 4.5a. Binary logistic regression of patient characteristics and colonic pathology associated with faecal calprotectin (cut-off 50µg/g)

Table 4.5b Binary logistic regression of patient characteristics and colonic pathology associated with faecal calprotectin (cut-off 200µg/g)

Characteristic	Univariate analysis OR (95% CI)	P-value	Multivariate analysis OR (95% CI)	P-value
Patient characteristic				
Age	1.15 (0.81-1.61)	0.436	-	-
Sex	0.92 (0.57-1.48)	0.724	-	-
SIMD	0.92 (0.78-1.07)	0.268	-	-
BMI	1.72 (1.03-2.87)	0.037	1.51 (0.89-2.57)	0.124
Smoking	0.88 (0.54-1.44)	0.613	-	-
Aspirin	1.02 (0.53-1.94)	0.955	-	-
NSAIDs	1.8 (0.95-3.39)	0.071	1.19 (0.6-2.3)	0.627
PPI	2.95 (1.80-4.84)	<0.001	3.16 (1.87-5.31)	<0.001
Statin	1.04 (0.64-1.7)	0.863	-	-
Metformin	1.61 (0.66-3.93)	0.298	-	-
ACE Inhibitor	0.83 (0.45-1.55)	0.562	-	-
Colonic Pathology				
Normal	1.17 (0.71-1.93)	0.543	-	-
Other	0.45 (0.69-2.29)	0.454	-	-
Inflammatory	2.01 (0.69-5.81)	0.198	-	-
Non-advanced adenoma	0.72 (0.38-1.35)	0.303	-	-
Advanced adenoma	0.61 (0.32-1.17)	0.137	-	-
Advanced neoplasia	0.74 (0.41-1.34)	0.320	-	-
CRC	1.65 (0.54-5.07)	0.379	-	-

Table 4.5b. Binary logistic regression of patient characteristics and colonic pathology associated with faecal calprotectin (cut-off 200µg/g)

Table 4.6 Sensitivity, specificity, PPV, NPV of faecal calprotectin

Category	n	Cut off ≥50 μg/g				Cut off ≥200 μg/g			
		Sens (%)	Spec (%)	PPV (%)	NPV (%)	Sens (%)	Spec (%)	PPV (%)	NPV (%)
Normal	116	58.9 52.3-65.2	38.8 29.9-48.3	66.2 62.1-70.1	31.7 26.1-37.9	24.6 19.2-30.6	72.4 63.4-80.3	64.4 55.6-72.4	32.1 29.2-35.0
Other	65	63.1 50.2-74.7	41.1 35.4-47.1	19.5 16.4-23.0	83.1 77.7-87.4	29.2 18.6-41.8	75.3 69.9-80.1	21.1 14.8-29.1	82.4 79.9-84.8
Inflammation	15	93.3 68.1-99.8	41.8 36.5-47.3	6.7 5.7-7.8	99.3 95.5-99.9	40.0 16.3-67.7	75.1 70.1-79.6	6.7 3.6-12.0	96.6 94.9-97.7
Non-advanced adenoma	72	48.6 36.7-60.7	37.5 31.8-43.5	16.7 13.4-20.5	73.9 68.4-78.8	20.8 12.2-32.0	73.2 67.6-78.3	16.7 10.9-24.6	78.2 75.8-80.5
Advanced adenoma	70	51.4 39.2-63.6	38.3 32.6-44.3	17.1 13.9-20.9	76.1 70.5-80.8	18.6 10.3-29.7	72.7 67.1-77.8	14.4 9.1-22.2	78.3 75.9-80.4
Advanced neoplasia	84	58.3 47.1-69.0	39.9 34.0-46.1	23.3 19.9-27.2	75.6 69.5-80.4	21.4 13.2-31.7	73.1 67.4-78.4	20.0 13.7-28.3	74.8 72.2-77.2
CRC	14	92.8 66.1-99.8	41.7 36.4-47.2	6.2 5.3-7.3	99.3 95.5-99.9	64.3 35.1-87.2	74.9 69.9-79.4	9.6 6.4-14.0	98.1 96.2-99.0

Table 4.6. Sensitivity, specificity, PPV, NPV of faecal calprotectin

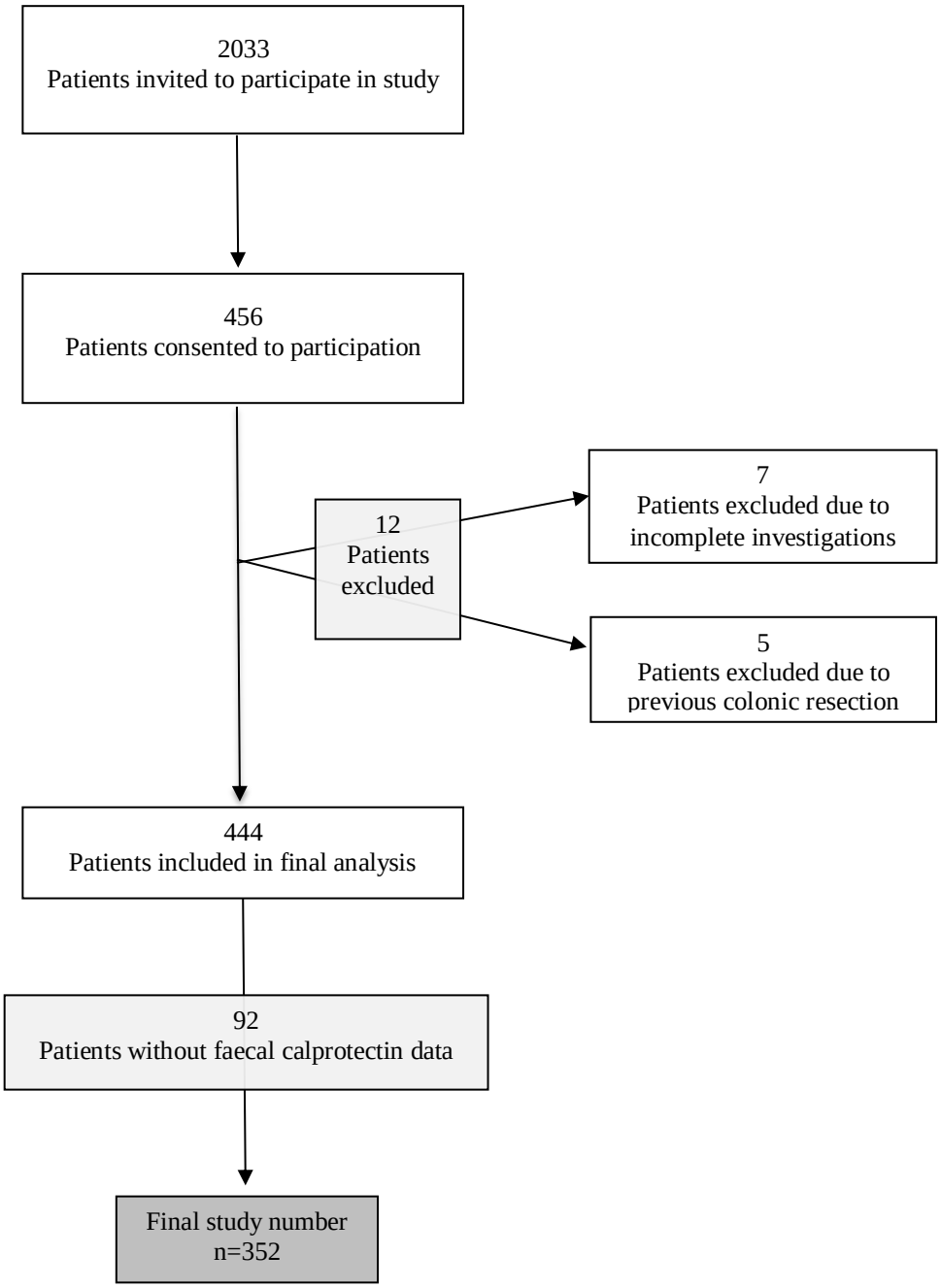

Figure 4.1. Flowchart of patient recruitment

5.0 The relationship between colonic inflammation, measures of systemic inflammation and the presence of neoplasia in a Scottish Bowel Screening Programme cohort

5.1 Introduction

The presence of an inflammatory tumour microenvironment has been recognised as the seventh hallmark of cancer. [173] The relationships between the presence of a systemic inflammatory response and colorectal cancer development and outcomes is well described. Systemic inflammation measured in peripheral blood is a validated, stage-independent predictor of poorer cancer outcomes in both early resectable colorectal cancer as well as advanced disease. [186, 187] Furthermore there is evidence that pre-diagnostic evidence of systemic inflammation is associated with future development of colorectal cancer. [170]

Within the tumour microenvironment, conflicting pro-tumour and anti-tumour inflammatory responses can dictate cancer outcomes. The presence of a higher stromal to epithelial volume within the tumour is associated with presence of immune-suppressive pro-cancer inflammation and poorer cancer outcomes. [148, 149] Conversely a pronounced local inflammatory response characterised by a high grade lymphocytic infiltrate within the tumour microenvironment is associated with improved cancer outcomes. [193, 147, 194]

Assessment of the patient's SIR can be measured by circulating biomarkers such as CRP, albumin and components of the white cell count (WCC). From this a number of inflammation-based prognostic scores have been developed including the mGPS. This is a score of 0-2 based on pre-operative CRP and albumin. In CRC the prognostic value of mGPS has been validated globally. [283, 284, 147, 159] NLR, has also been shown to have prognostic value in colorectal cancer. [284] Given their ease and frequency of routine measurement it is relatively easy to put SIR into clinical practice in the management of colorectal cancer.

There are numerous tumour microenvironment characteristics and scores which have been developed to assess the local lymphocytic responses including the Immunoscore and KM grade. Previous studies have already investigated the relationship between the SIR and the local inflammatory response in colorectal cancer using these measures. [164, 165, 159, 285, 161] A limiting factor for introduction of these measures into clinical practice is that these measures of peritumoural inflammation required histological evaluation of primarily resection specimens.

Calprotectin detected in the faeces is a sensitive measure of intraluminal colonic inflammation and is currently well established in the clinical assessment of IBD [206]. High levels of FC have also been found to occur in malignancy. [234, 255] In Chapter 4, I investigated the relationship

between FC and the presence of colorectal neoplasia, demonstrating a relationship between elevated FC and presence of CRC. Patients with CRC had a median FC 138.5µg/g (compared to 81.0 µg/g in patients without CRC) and more than 90% has an elevated FC >50µg/g ($p<0.05$). FC was highly sensitive to CRC (92.9% 95% CI 66.1-99.8), at a cut off of 50µg/g, but with low specificity. There was no similar significant relationship observed between FC and non-invasive colorectal neoplasia.

The relationship between intraluminal colonic inflammation (measured using FC) and systemic inflammation in the context of a colorectal cancer screening population has not previously been evaluated. It is not clear whether assessment of the SIR in FIT positive patients could refine the screening process, selecting high or low risk patients for presence of neoplasia. Furthermore, the combination of FC and SIR may also be of value in this regard.

In this chapter I aim to investigate associations between systemic inflammation, clinical, and pathological characteristics of patients undergoing colonoscopy via the SBSP. I will also specifically investigate the relationship between measures of the systemic inflammatory response and luminal inflammation measured using FC, with the hypothesis that an elevated faecal calprotectin is associated with the presence of a systemic inflammatory response.

5.2 Methods

5.2.1 Patients and Methods

'Investigation of the local and systemic inflammatory response and of dietary habits in those attending for investigation via the National Health Service Colorectal Cancer Screening Programme' was a study set up to examine multiple research questions including the inflammatory response of those attending for colonoscopy via the SBSP (see study protocol - Appendix a.)

Patients for this chapter were identified from this study, which is the same population examined in Chapter 4. The full methodology can therefore be found in Chapter 4.2.2.

The cohort of patients used in the final analysis was sub-divided based on the final pathology results to allow comparison of neoplastic and inflammatory conditions. The categories are listed in Table 5.1.

5.2.2 Inflammatory Markers

As part of this study, faecal calprotectin measurements, and blood for marker of systemic inflammation were obtained.

Consenting patients provided a stool sample for calprotectin, taken prior to commencement of bowel preparation. They returned the sample on the day of colonoscopy. Faecal calprotectin was analysed by the NHS biochemistry laboratory, at Glasgow Royal infirmary using standard clinical grade assays (Bühlmann fCAL® ELISA). Faecal calprotectin was divided into 3 categories for analysis based upon local thresholds; <50µg/g is deemed normal, but only >200µg/g is deemed clinically significant.[222] This resulted in 3 groups: <50µg/g, 50-200µg/g and >200 µg/g.

Blood tests were obtained at the time of IV cannula insertion, (sited for sedation at the time of colonoscopy), for full blood count (FBC), C-reactive protein (CRP), and albumin. White cell count was divided into individual components including neutrophils and lymphocytes. Three markers of SIR were used for analysis; NLR, CRP and mGPS. NLR is an inflammatory biomarker calculated by dividing the absolute number of neutrophils by the absolute number of lymphocytes, to display neutrophilia accompanied by a relative lymphocytopaenia in SIR. [195] NLR thresholds vary, with NLR >5 being associated with reduced 5 year overall and cancer specific survival in CRC. [196] More recently an NLR more than the median of ~2.74 is reported to be associated with reduced disease specific survival. [197] NLR <3 is the cut-off used in this study. CRP is an acute phase protein which rises in response to acute inflammation and tissue damage. CRP >10mg/l is the routine detection reference limit for an elevated CRP. [286] However a CRP of >3 mg/l has been associated with increased risk of early cancer death. [286, 196] CRP <3 is the cut-off used in this study. mGPS is calculated based on CRP and albumin levels. A CRP increase (> 10 mg/L) and

hypoalbuminemia (< 35 g/L) gives a score of 2. Patients with increased CRP, but a normal albumin gives a score of 1. Patients with normal CRP and albumin gives a score of 0. [196]

5.2.3 Objectives

The primary objective was to analyse associations between systemic inflammation, clinical, and pathological characteristics of patients undergoing colonoscopy via the SBSP. Secondary objective to analyse the relationship between measures of the systemic inflammatory response and luminal inflammation measured using FC, with the hypothesis that an elevated faecal calprotectin is associated with the presence of a systemic inflammatory response.

5.2.4 Statistical Analysis

Percentages are rounded to the nearest whole number, and therefore total may not be 100%. Categorical data regarding patient characteristics were compared using the Chi square test and Chi square test for linear association where appropriate. Faecal calprotectin results were expressed as medians, and analysed using non-parametric Kruskal-Wallis. Binary logistic regression was used to examine the relationship between patient factors and SIR, and calculate a HR with 95% CI. Factors on univariate analysis with a p value <0.10 were taken into a multivariate model using a backward conditional model to identify independently significant factors. A p value of <0.05 was considered significant for all analysis. Statistical analysis was performed using SPSS version 25.0 for Windows (IBM Corporation, Armonk, NY).

5.2.5 Ethics

This study was approved by the NHS Greater Glasgow and Clyde Research and Development department, and the West of Scotland Research Ethics Service (REC number: 15/WA/0053).

5.3 Results

2033 patients were invited to participate in the study. 456 patients consented to participate, returning the relevant paperwork. Figure 5.1 details the final cohort, with 12 patients excluded (seven due to incomplete investigations, and five whom had undergone previous colonic resection). Therefore 444 patients are included in the final analysis.

5.3.1 Patient Characteristics

Table 5.2 displays the patient characteristics. Two hundred and thirty five patients (53%) were female, and two hundred and five (46%) were between age of 60-69 years. One hundred and thirty two (30%) were in the lowest (most deprived) Scottish Index of Multiple Deprivation (SIMD) quintile. One hundred and fifty three (38%) were obese with a BMI 30+. Two hundred and nineteen (52%) had ever smoked. Three hundred and fifty two (79%) patients returned a faecal calprotectin sample and ninety (26%) patients had a FC more than 200. A measurement of SIR was recorded in more than 80% of the cohort. Two hundred and forty eight (67%) had a CRP ≥3, one hundred and seventy three (42%) had an NLR ≥3 and only forty seven (13%) had an elevated mGPS (1-2). The relationship between patient characteristics and pathology is described in Table 5.9.

5.3.2 Systemic inflammatory response and patient characteristics

In this SBSP population patient characteristics of age, sex, SIMD quintile, BMI, smoking status and medication use were examined in relation to markers of SIR. Table 5.3a details their associations with CRP. Higher BMI, lower SIMD quintile, smokers, aspirin and PPI use were associated with a higher CRP. Table 5.3b details associations with mGPS. Higher BMI, NSAID and PPI use and patients not on a statin were associated with higher mGPS. Table 5.3c details association with NLR. Older age and lower BMI were associated with higher NLR

All of the markers of SIR were examined for an association with the patient characteristics using binary logistic regression.

The relationships between individual clinicopathological characteristics and elevated CRP are examined in Table 5.4a. Decreasing deprivation (OR 0.84, 95% CI 0.71-0.99, $p<0.05$) is inversely associated with CRP ≥3. BMI (OR 3.49, 95%CI 2.00-6.09, $p<0.001$), smoking (OR 2.30, 95% CI 1.36-3.88, $p<0.05$) and aspirin use (OR 2.51, 95%CI 1.12-5.59, $p<0.05$) were independently positively associated with CRP ≥3.

The relationship between patient characteristics and mGPS was examined in Table 5.4b. BMI (OR 3.36, 95%CI 1.61-7.02, p 0.001) and NSAID use (OR 2.72, 95%CI 1.23-6.06, $p<0.05$) were

independently positively associated with mGPS. Statin use (OR 0.18, 95%CI 0.07-0.45, p<0.001) was independently negatively associated with mGPS.

The relationship between NLR and patient characteristics was examined in Table 5.4c. Age (OR 1.5 95% CI 1.1-2.03, p<0.05) and ACE inhibitor use (OR 1.72, 95% CI 1.02-2.9, p<0.05) were independently positively associated with NLR. BMI (OR 0.55, 95% CI 0.35-0.86, p<0.01) was negatively associated with NLR.

5.3.3 Systemic inflammatory response and colorectal pathology

Next I reviewed whether there is a relationship between SIR and colorectal pathology diagnosed at screening colonoscopy. The relationships are demonstrated in Tables 5.5a-c.

21 patients were diagnosed with inflammatory pathology. These patients were more likely to be systemically inflamed compared to patients with normal colonoscopy (86% vs. 68%, p=0.065). There were no other significant relationships between markers of the SIR and pathology category.

5.3.4 Systemic inflammatory response and luminal/local inflammation (FC)

Tables 5.6 and 5.7 display the association of faecal calprotectin with markers of SIR. In this cohort, there was no significant relationship between FC and markers of SIR. With the exception of white cell count, all other markers of SIR (CRP, NLR, mGPS, neutrophils and lymphocytes) had a higher median FC in higher levels of SIR, but did not reach statistical significance.

5.3.5 Overall inflammation and colorectal pathology

To allow comparison of overall inflammation with colorectal pathology, a marker of the SIR (CRP) was combined with a marker of the LIR (FC). Table 5.8a displays the four categories; 1: low LIR and SIR, 2: low LIR and high SIR, 3: high LIR and low SIR, and 4: high LIR and SIR.

Table 5.8b displays the relationship between pathology and overall inflammatory response. Seventy-six (25%) of patients were in category1, one hundred and forty six (48%) in category 2, twenty-two (7%) in category 3 and sixty two (20%) in category 4. Patients with inflammatory pathology had the highest percentage (40%, n=6) of patients in category 4, and the lowest percentage (13%, n=2) of patients in category 1. Patients with CRC had an equal distribution among the first two categories, but a higher percentage (39%, n=5) of patients were in category 4 with high SIR and LIR. Patients with non-cancer neoplasia had similar distribution through the categories as those with no pathology.

5.4 Discussion

This chapter aimed to investigate the relationships between systemic inflammation, luminal inflammation and patient clinical and pathological characteristics in those undergoing colonoscopy via the Scottish Bowel Screening Programme.

In this cohort CRC was shown to be associated with increasing age. Age is a known risk factor for CRC, as evidenced by the SBSP starting at age 50. Other known risk factors for CRC include higher BMI and smoking. However this association was not demonstrated in this cohort. Given the tangibility of these known risk factors, this is likely due to the combination of the small numbers of CRCs diagnosed in this study and fact that patients who participate in cancer screening are not representative of the population as a whole, often making more health-conscious decisions. [280] Therefore this is a different cohort of patients from the symptomatic patients more commonly included in FC studies.

Patients with inflammatory pathology were associated with a rise in SIR – both CRP and NLR. No other pathology group was associated with SIR, and in particular patients with CRC did not demonstrate an associated raised SIR. CRC is known to have an association with inflammation, however this was not reflected in this cohort of patients. This may be due to the small numbers of CRC diagnosed. Although data on stage was not available, CRC diagnosed through bowel screening is often at an earlier stage which itself is less likely to be associated with a SIR. Indeed this may explain why in this screening cohort there is no connection between CRC and SIR. The cut-off points of the markers of SIR were also set based upon levels that have shown to be positive in cancer/inflammation, however at the lower end of this range. It may be that a higher cut-off point is more discriminatory in a screening cohort.

In this population there are multiple associations between the three markers of inflammation and patient characteristics. However no single characteristic links with all of the markers of the SIR. In an unselected bowel screening cohort there are a multitude of confounding factors interplaying with results e.g. co-morbidities, medication use. The variation does suggest that there is no superior marker of systemic inflammation in this context, and that a multitude of factors work together to influence the SIR.

No significant relationship between FC and SIR was demonstrated. Patients with a FC greater than 200 had a higher median CRP and NLR, in comparison to those with a lower FC. There is limited literature on the relationship between FC and SIR in CRC. In 1998, Kristinsson et al. found no significant correlation in colorectal cancer between FC and markers of systemic inflammation (CRP, CEA, plasma calprotectin). [249]

I have previously used the same cohort of patients to study the relationship between FC and colorectal neoplasia, in more detail. This showed a relationship between FC and CRC (as well as the well-studied inflammatory pathology), with no significant relationship observed between FC and non-invasive colorectal neoplasia.

There are a number of limitations with this study. The patients in this study are selected from a FOBT positive bowel screening population. As discussed above patients who participate in cancer screening are not representative of the population as a whole, [280] making this a different cohort from both the general population and symptomatic patients more commonly included in FC studies. Another potential limitation is the timing of analysis of FC. Patients obtained their sample at least 24 hours prior to returning it for analysis (due to the timing of bowel preparation). This reflects working clinical practice and stool samples for FC can be stored for up to 3 days. [216, 217] However it is obviously preferable to analyse the sample as soon as possible, as any delays in time of analysis could affect the results. The sample size was calculated based on power calculations for the original study, with the aim of investigating the impact of dietary components on the colonic health of people who present via the Scottish Bowel Screening Programme, however the low specificity reported in our study would not support a larger powered study.

More work is required to fully assess how FC, SIR and colonic pathology intertwine, particularly in colorectal cancer, to help understand the potential role FC can play in diagnosis or management. The current data do not suggest that what we know about the relationship between cancer and inflammation can be extrapolated to the wider screening population. Therefore we cannot use the standard levels of SIR as markers of pathology in the diagnosis of organic bowel disease. Given the association between CRC and inflammation (both systemic and local as demonstrated by FC) an association between these markers of the SIR and luminal inflammation would be expected. However, given the opposite prognostic values of a high LIR and high SIR, in CRC, it may be that low levels of FC reflect a low LIR and the reason for heterogeneity of FC in CRC. It is also unclear what component of the LIR is measured by FC. As discussed previously FC is released upon neutrophil cell death and may therefore be a measurement of a neutrophilic LIR. Whereas it is a lymphocytic LIR which has most recently been shown to confer good prognosis. [166, 201] More in-depth study of the LIR in patients with a high FC is required to understand this.

In conclusion, this study has shown that while there are many patient factors associated with the systemic inflammatory response and colonic pathology, these relationships are not consistent. As such, current measures of the SIR may add little to the identification of high-risk patients in a bowel screening population.

5.5 Tables and Figures

Table 5.1 Patient Categories	
Normal	No pathology
Other	Non-inflammatory, non-neoplastic pathology i.e. diverticulosis, haemorrhoids
Inflammatory	Active inflammatory conditions i.e. colitis, diverticulitis
Neoplastic	
Non-advanced adenoma	Adenoma without advanced features
Advanced adenoma	Adenoma with one advanced feature of high grade dysplasia, ≥ 1cm at pathology, villous
Advanced neoplasia	Advanced adenoma and/or malignancy
Cancer	Malignancy only

Table 5.1 Patient Categories

Table 5.2. Clinical characteristics	
Characteristic	All patients n (%)
	444
Age (years)	
<60	172 (39)
60-69	205 (46)
70+	67 (15)
Sex	
Female	235 (53)
Male	209 (47)
SIMD Quintile	
1	132 (30)
2	74 (17)
3	71 (16)
4	72 (16)
5	95 (21)
BMI (403)[a]	
<30	250 (62)
30+	153 (38)
Smoking (423)[a]	
Never	204 (48)
Ever	219 (52)
Drugs (number of patients on) (443)[a]	
Aspirin	74 (17)
NSAIDs	68 (15)
PPI	185 (41)
Statin	175 (40)
Metformin	31 (7)
ACE	89 (20)
Faecal Calprotectin (352)[a]	
<50	142 (40)
50-200	120 (34)
>200	90 (26)
CRP (368)[a]	
<3	120 (33)
≥3	248 (67)
NLR (410)[a]	
<3	237 (58)
≥3	173 (42)
mGPS (366)[a]	
0	319 (87)
1/2	47 (13)
Bowel screening investigation outcome (444)	
Normal	139 (31)
Other	83 (19)
Inflammatory	24 (5)
Non-advanced adenoma	87 (20)
Advanced adenoma	92 (21)
Advanced neoplasia	111 (25)
CRC	19 (4)

Table 5.2 Clinical Characteristics
SIMD: Scottish Index of Multiple Deprivation BMI: body mass index NSAIDS: non-steroidal anti-inflammatory drugs, PPI: proton pump inhibitors, CRP: C - reactive protein, NLR: neutrophil lymphocyte ratio, NNNICP: non-neoplastic non-inflammatory colorectal pathology, NNICP: non-neoplastic inflammatory colorectal pathology, CRC: colorectal cancer
a Number of patients when incomplete data available

Table 5.3a. Relationship between patient characteristics and SIR - CRP

		n (%)[a]	CRP <3 n (%)	CRP ≥3 n (%)	P-value*
All patients		368	120 (33)	248 (67)	-
Age (years)					
<60		141 (38)	51 (36)	90 (64)	0.592
60-69		174 (47)	50 (29)	124 (71)	
70+		53 (14)	19 (36)	34 (64)	
Sex					
Female		196 (53)	68 (35)	128 (65)	0.362
Male		172 (47)	52 (30)	120 (70)	
SIMD Quintile					
1		105 (29)	27 (26)	78 (74)	0.002
2		60 (16)	14 (23)	46 (77)	
3		62 (17)	20 (32)	42 (68)	
4		61 (17)	23 (38)	38 (62)	
5		80 (22)	36 (45)	44 (55)	
BMI					
<30		205 (62)	87 (42)	118 (58)	<0.001
30+		128 (38)	24 (19)	104 (81)	
Smoking					
Never		171 (49)	72 (42)	99 (58)	<0.001
Ever		179 (51)	42 (24)	137 (77)	
Drugs					
Aspirin	N	304 (83)	110 (36)	194 (64)	0.001
	Y	63 (17)	9 (14)	54 (86)	
NSAIDs	N	310 (84)	104 (34)	206 (67)	0.284
	Y	57 (16)	15 (26)	42 (74)	
PPI	N	214 (58)	82 (38)	132 (62)	0.004
	Y	153 (42)	37 (24)	116 (76)	
Statin	N	221 (60)	73 (33)	148 (67)	0.760
	Y	146 (40)	46 (32)	100 (69)	
Metformin	N	340 (93)	111 (33)	229 (67)	0.747
	Y	27 (7)	8 (30)	19 (70)	
ACE	N	295 (80)	100 (34)	195 (66)	0.222
	Y	72 (20)	19 (26)	53 (74)	

Table 5.3a. Relationship between patient characteristics and SIR – CRP

[a] Number of patients when incomplete data available ∗chi squared

Table 5.3b. Relationship between patient characteristics and SIR - mGPS

		n (%) [a]	mGPS 0 n (%)	mGPS 1/2 n (%)	P-value*
All patients		366	319 (87)	47 (13)	-
Age (years)					
<60		139 (38)	123 (89)	16 (12)	0.642
60-69		174 (48)	150 (86)	24 (14)	
70+		53 (14)	46 (87)	7 (13)	
Sex					
Female		195 (53)	172 (88)	23 (12)	0.523
Male		171 (47)	147 (86)	24 (14)	
SIMD Quintile					
1		105 (29)	91 (87)	14 (13)	0.585
2		60 (16)	52 (87)	8 (13)	
3		62 (17)	51 (82)	11 (18)	
4		60 (16)	56 (93)	4 (7)	
5		79 (22)	69 (87)	10 (13)	
BMI					
<30		204 (62)	189 (93)	15 (7)	0.001
30+		127 (38)	102 (80)	25 (20)	
Smoking					
Never		170 (49)	145 (85)	25 (15)	0.335
Ever		178 (51)	158 (89)	20 (11)	
Drugs					
Aspirin	N	302 (83)	259 (86)	43 (14)	0.089
	Y	63 (17)	59 (94)	4 (6)	
NSAIDs	N	309 (85)	277 (90)	32 (10)	0.001
	Y	56 (15)	41 (73)	15 (27)	
PPI	N	212 (58)	192 (91)	20 (9)	0.021
	Y	153(42)	126 (82)	27 (18)	
Statin	N	219 (60)	183 (84)	36 (16)	0.013
	Y	146 (40)	135 (93)	11 (8)	
Metformin	N	338 (93)	297 (88)	41 (12)	0.132
	Y	27 (7)	21 (78)	6 (22)	
ACE	N	293 (80)	252 (86)	41 (14)	0.199
	Y	72 (20)	66 (92)	6 (8)	

Table 5.3b. Relationship between patient characteristics and SIR – mGPS

[a] Number of patients when incomplete data available ∗chi squared linear by linear

Table 5.3c Relationship between patient characteristics and SIR - NLR

		NLR <3 n (%)	NLR ≥3 n (%)	P-value*
All patients (410)[a]		237 (58)	173 (42)	-
Age (years)				
<60		104 (66)	53 (34)	0.002
60-69		106 (55)	86 (45)	
70+		27 (44)	34 (56)	
Sex				
Female		131 (61)	84 (39)	0.178
Male		106 (54)	89 (46)	
SIMD Quintile				
1		77 (64)	44 (36)	0.108
2		37 (55)	30 (45)	
3		39 (61)	25 (39)	
4		37 (54)	31 (46)	
5		47 (52)	43 (48)	
BMI (372)[a]				
<30		123 (53)	108 (47)	0.007
30+		95 (67)	46 (33)	
Smoking (390)[a]				
Never		115 (61)	74 (39)	0.305
Ever		112 (56)	89 (44)	
Drugs				
Aspirin	N	193 (57)	146 (43)	0.361
	Y	44 (63)	26 (37)	
NSAIDs	N	202 (58)	145 (42)	0.796
	Y	35 (57)	27 (44)	
PPI	N	143 (59)	99 (41)	0.572
	Y	94 (56)	73 (44)	
Statin	N	148 (60)	98 (40)	0.265
	Y	89 (55)	74 (45)	
Metformin	N	222 (58)	159 (42)	0.627
	Y	15 (54)	13 (46)	
ACE	N	197 (60)	131 (40)	0.081
	Y	40 (49)	41 (51)	

Table 5.3c Relationship between patient characteristics and SIR – NLR

[a] Number of patients when incomplete data available *chi squared linear by linear

Table 5.4a. Binary logistic regression of patient characteristics associated with CRP (cut-off 3)

Characteristic	Univariate analysis OR (95% CI)	P-value	Multivariate analysis OR (95% CI)	P-value
Age	1.09 (0.79-1.50)	0.592	-	-
Sex	1.23 (0.79-1.90)	0.363	-	-
SIMD	0.79 (0.69-0.92)	0.002	0.84 (0.71-0.995)	0.044
BMI	3.20 (1.89-5.39)	<0.001	3.49 (2.00-6.09)	<0.001
Smoking	2.37 (1.50-3.76)	<0.001	2.30 (1.36-3.88)	0.002
Aspirin	3.40 (1.62-7.16)	0.001	2.51 (1.12-5.59)	0.025
NSAIDs	1.41 (0.75-2.67)	0.285	-	-
PPI	1.95 (1.23-3.09)	0.005	1.51 (0.89-2.57)	0.123
Statin	1.07 (0.69-1.68)	0.760	-	-
Metformin	1.15 (0.49-2.71)	0.747	-	-
ACE Inhibitor	1.43 (0.80-2.55)	0.224	-	-

Table 5.4a Binary logistic regression of patient characteristics associated with CRP (cut-off 3)

Table 5.4b. Binary logistic regression of patient characteristics associated with mGPS (cut-off 0)

Characteristic	Univariate analysis OR (95% CI)	P-value	Multivariate analysis OR (95% CI)	P-value
Age	1.11 (0.71-1.73)	0.641	-	-
Sex	1.22 (0.66-2.25)	0.523	-	-
SIMD	0.95 (0.77-1.16)	0.585	-	-
BMI	3.09 (1.56-6.12)	0.001	3.36 (1.61-7.02)	0.001
Smoking	0.73 (0.39-1.38)	0.336	-	-
Aspirin	0.41 (0.14-1.18)	0.099	0.39 (0.80-1.86)	0.235
NSAIDs	3.17 (1.58-6.35)	0.001	2.72 (1.23-6.06)	0.014
PPI	2.06 (1.11-3.83)	0.023	2.09 (0.97-4.51)	0.060
Statin	0.41 (0.20-0.84)	0.015	0.18 (0.07-0.45)	<0.001
Metformin	2.07 (0.79-5.43)	0.139	-	-
ACE Inhibitor	0.56 (0.23-1.37)	0.204	-	-

Table 5.4b Binary logistic regression of patient characteristics associated with mGPS (cut-off 0)

Table 5.4c. Binary logistic regression of patient characteristics associated with NLR (cut-off 3)

Characteristic	Univariate analysis OR (95% CI)	P-value	Multivariate analysis OR (95% CI)	P-value
Age	1.58 (1.18-2.10)	0.002	1.50 (1.1-2.03)	0.010
Sex	1.31 (0.88-1.94)	0.179	-	-
SIMD	1.11 (0.98-1.26)	0.108	-	-
BMI	0.55 (0.36-0.85)	0.008	0.55 (0.35-0.86)	0.009
Smoking	1.24 (0.83-1.85)	0.305	-	-
Aspirin	0.78 (0.46-1.33)	0.361	-	-
NSAIDs	1.08 (0.63-1.85)	0.796	-	-
PPI	1.12 (0.75-1.67)	0.572	-	-
Statin	1.26 (0.84-1.87)	0.265	-	-
Metformin	1.21 (0.56-2.61)	0.627	-	-
ACE Inhibitor	1.54 (0.95-2.51)	0.082	1.72 (1.02-2.9)	0.041

Table 5.4c. Binary logistic regression of patient characteristics associated with NLR (cut-off 3)

Table 5.5a. Relationship between pathology and systemic inflammatory response – CRP

Categories	n[a]	CRP <3 n (%)	CRP ≥ 3 n (%)	P-value*
All Patients	368	120 (33)	248 (67)	-
Normal	108	35 (32)	73 (68)	0.958
Other	69	27 (39)	42 (61)	0.200
Inflammatory	21	3 (14)	18 (86)	0.065
Non-advanced adenoma	74	21 (28)	53 (72)	0.385
Advanced adenoma	79	28 (35)	51 (65)	0.544
Advanced neoplasia	96	34 (35)	62 (65)	0.495
CRC	17	6 (35)	11 (65)	0.809

Table 5.5a Relationship between pathology and systemic inflammatory response – CRP.
a Number of patients when incomplete data available *chi squared linear by linear

Table 5.5b. Relationship between pathology and systemic inflammatory response - mGPS

Categories	n[a]	mGPS 0 n (%)	mGPS 1/2 n (%)	P-value*
All Patients	366	319 (87)	47 (13)	-
Normal	107	92 (86)	15 (14)	0.665
Other	69	62 (90)	7 (10)	0.457
Inflammatory	20	16 (80)	4 (20)	0.325
Non-advanced adenoma	74	64 (87)	10 (14)	0.847
Advanced adenoma	79	70 (89)	9 (11)	0.664
Advanced neoplasia	96	85 (89)	11 (12)	0.637
CRC	17	15 (88)	2 (12)	0.892

Table 5.5b Relationship between pathology and systemic inflammatory response – mGPS
[a] Number of patients when incomplete data available *chi squared linear by linear

Table 5.5c. Relationship between pathology and systemic inflammatory response - NLR

Categories	n[a]	NLR <3 n (%)	NLR≥3 n (%)	P-value*
All Patients	410	237 (58)	173 (42)	-
Normal	127	79 (62)	48 (38)	0.227
Other	77	46 (60)	31 (40)	0.703
Inflammatory	23	8 (35)	15 (65)	0.021
Non-advanced adenoma	78	45 (58)	33 (42)	0.982
Advanced adenoma	86	45 (52)	41 (48)	0.247
Advanced neoplasia	105	59 (56)	46 (44)	0.698
CRC	19	14 (74)	5 (26)	0.151

Table 5.5c. Relationship between pathology and systemic inflammatory response – NLR
[a] Number of patients when incomplete data available *chi squared linear by linear

Table 5.6 Relationship between faecal calprotectin and the systemic inflammatory response

	n[a]	Median FC (IQR)	P-value	FC <50 n (%)	FC 50-200 n (%)	FC >200 n (%)	P-value*
All	352	0.773	-	142 (40)	120 (34)	90 (26)	-
CRP	306						
<3	98	78.0 (29.0-176.5)	0.773	43 (44)	33 (34)	22 (22)	0.183
≥3	208	81.0 (29.0-244.0)		79 (38)	67 (32)	62 (30)	
NLR	337						
<3	192	77.5 (29.0-207.0)	0.999	75 (39)	69 (36)	48 (25)	0.976
≥3	145	78.0 (29.0-222.5)		61 (42)	43 (30)	41 (28)	
mGPS	305						
0	268	78.0 (29.0-223.8)	0.212	108 (40)	87 (33)	73 (27)	0.591
1/2	37	108.0 (35.5-243.0)		13 (35)	13 (35)	11 (30)	
WCC	338						
<8.5	257	69.0 (29.0-212.5)	0.435	106 (41)	85 (33)	66 (26)	0.625
8.5-11	60	102.0 (32.5-215.0)		20 (33)	24 (40)	16 (27)	
>11	21	66.0 (29.0-236.5)		10 (48)	4 (19)	7 (33)	
Neutrophils	337						
<7.5	304	77.5 (29.0-210.8)	0.757	123 (41)	103 (34)	78 (26)	0.554
≥7.5	33	97.0 (29.0-236.5)		13 (39)	9 (27)	11 (33)	
Lymphocytes	337						
<1	25	51.0 (29.0-327.5)	0.939	12 (48)	5 (20)	8 (32)	0.752
1-3	297	78.0 (29.0-212.5)		118 (40)	103 (35)	76 (26)	
>3	15	84.0 (37.0-256.0)		6 (40)	4 (27)	5 (33)	

Table 5.6 Relationship between faecal calprotectin and the systemic inflammatory response

[a] Number of patients when incomplete data available *chi squared linear by linear

Table 5.7 Relationship between faecal calprotectin and the systemic inflammatory response

All patients		FC	CRP	NLR	mGPS	WCC	Neutrophils	Lymphocytes
FC	Pearson Corr							
	Sig							
	N							
CRP	Pearson Corr	0.059						
	Sig	0.308						
	N	368						
NLR	Pearson Corr	-0.023	0.108					
	Sig	0.675	0.041					
	N	337	359					
mGPS	Pearson Corr	0.067	0.710	0.081				
	Sig	0.241	<0.01	0.125				
	N	305	366	357				
WCC	Pearson Corr	-0.055	0.273	0.353	0.196			
	Sig	0.310	<0.01	<0.01	<0.01			
	N	338	360	410	358			
Neutrophils	Pearson Corr	-0.057	0.265	0.618	0.191	0.931		
	Sig	0.295	<0.01	<0.01	<0.01	<0.01		
	N	337	359	410	357	410		
Lymphocytes	Pearson Corr	-0.052	0.065	-0.550	0.038	0.460	0.133	
	Sig	0.340	0.221	<0.01	0.475	<0.01	0.007	
	N	337	359	410	357	410	410	

Table 5.7 Relationship between faecal calprotectin and the systemic inflammatory response

[a] Number of patients when incomplete data available

Table 5.8a Overall inflammation coding	
1	Low LIR/ Low SIR
2	Low LIR/ High SIR
3	High LIR/ Low SIR
4	High LIR/ High SIR

Table 5.8a Overall Inflammation Coding
Low LIR: FC<200
High LIR: FC ≥200
Low SIR: CRP<3
High SIR: CRP ≥3

Table 5.8b Relationship between pathology and overall inflammatory response

	n (%)	Normal n (%)	Other n (%)	Inflammatory n (%)	Non-advanced adenoma n (%)	Advanced adenoma n (%)	Advanced neoplasia n (%)	CRC n (%)
1	76 (25)	22 (23)	17 (30)	2 (13)	14 (22)	17 (27)	21(28)	4 (31)
2	146 (48)	45 (47)	23 (41)	7 (47)	35 (55)	32 (52)	36 (48)	4 (31)
3	22 (7)	9 (9)	5 (9)	0 (0)	3 (5)	5 (8)	5 (7)	0 (0)
4	62 (20)	20 (21)	11 (20)	6 (15)	12 (19)	8 (13)	13 (17)	5 (39)

Table 5.8b Relationship between pathology and overall inflammatory response

Table 5.9. Relationship between patient characteristics and pathology

Characteristic		All Patients	Normal	P-value	Other	P-value	Inflam-matory	P-value	Non-advanced adenoma	P-value	Advanced adenoma	P-value	CRC	P-value
Age (years)														
<60		172 (39)	71 (51)	<0.01	31 (37)	0.246	11 (46)	0.316	26 (30)	0.012	31 (34)	0.767	2 (11)	0.004
60-69		205 (46)	58 (42)		34 (41)		11 (46)		41 (47)		50 (54)		11 (58)	
70+		67 (15)	10 (7)		18 (22)		2 (8)		20 (23)		11 (12)		6 (32)	
Sex														
Female		235 (53)	62 (45)	0.018	50 (60)	0.139	8 (33)	0.048	55 (63)	0.032	51 (55)	0.588	9 (47)	0.620
Male		209 (47)	77 (55)		33 (40)		16 (67)		32 (37)		41 (45)		10 (53)	
SIMD														
1		132 (30)	48 (35)	0.092	26 (30)	0.704	5 (21)	0.053	21 (24)	0.188	29 (32)	0.528	3 (16)	0.267
2		74 (17)	24 (17)		12 (15)		1 (4)		18 (21)		16 (17)		3 (16)	
3		71 (16)	21 (15)		13 (16)		7 (29)		11 (13)		15 (16)		4 (21)	
4		72 (16)	21 (15)		19 (23)		1 (4)		12 (14		14 (15)		5 (26)	
5		95 (21)	25 (18)		13 (16)		10 (42)		25 (29)		18 (20)		4 (21)	
BMI (403)[a]														
<30		250 (62)	75 (61)	0.669	42 (55)	0.132	17 (71)	0.360	57 (71)	0.058	49 (60)	0.634	10 (63)	0.969
30+		153 (38)	49 (40)		35 (46)		7 (29)		23 (29)		33 (40)		35 (38)	
Smoking (423)[a]														
Never		204 (48)	76 (58)	0.007	37 (46)	0.694	8 (35)	0.185	31 (37)	0.027	43 (49)	0.893	9 (50)	0.878
Ever		219 (52)	55 (42)		43 (54)		15 (65)		52 (63)		45 (51)		9 (50)	
Drugs (443)[a]														
Aspirin	N	369 (83)	114 (82)	0.625	64 (77)	0.094	21 (88)	0.570	72 (83)	0.881	81 (89)	0.101	17 (90)	0.461
	Y	74 (17)	25 (18)		19 (23)		3 (13)		15 (17)		10 (11)		2 (11)	
NSAIDs	N	375 (85)	107 (77)	0.002	67 (81)	0.271	21 (88)	0.690	80 (92)	0.035	82 (90)	0.105	18 (95)	0.212
	Y	68 (15)	32 (23)		16 (19)		3 (13)		7 (8)		9 (10)		1 (5)	
PPI	N	258 (58)	66 (48)	0.002	43 (52)	0.187	15 (63)	0.663	54 (62)	0.419	63 (69)	0.017	17 (90)	0.005
	Y	185 (42)	73 (53)		40 (48)		9 (38)		33 (38)		28 (31)		2 (11)	
Statin	N	268 (61)	89 (64)	0.304	43 (52)	0.072	19 (79)	0.054	44 (51)	0.035	59 (65)	0.342	14 (74)	0.229
	Y	175 (40)	50 (36)		40 (48)		5 (21)		43 (49)		32 (35)		5 (26)	
Metformin	N	412 (93)	130 (94)	0.770	75 (90)	0.295	24 (100)	0.167	79 (91)	0.370	86 (95)	0.528	18 (95)	0.762
	Y	31 (7)	9 (7)		8 (10)		0 (0)		8 (9)		5 (6)		1 (5)	
ACE	N	354 (80)	113 (81)	0.623	60 (72)	0.055	20 (83)	0.667	69 (79)	0.876	75 (82)	0.503	17 (90)	0.288
	Y	89 (20)	26 (19)		23 (28)		4 (17)		18 (21)		16 (18)		2 (11)	

Table 5.9 Relationship between patient characteristics and pathology
[a] Number of patients when incomplete data available

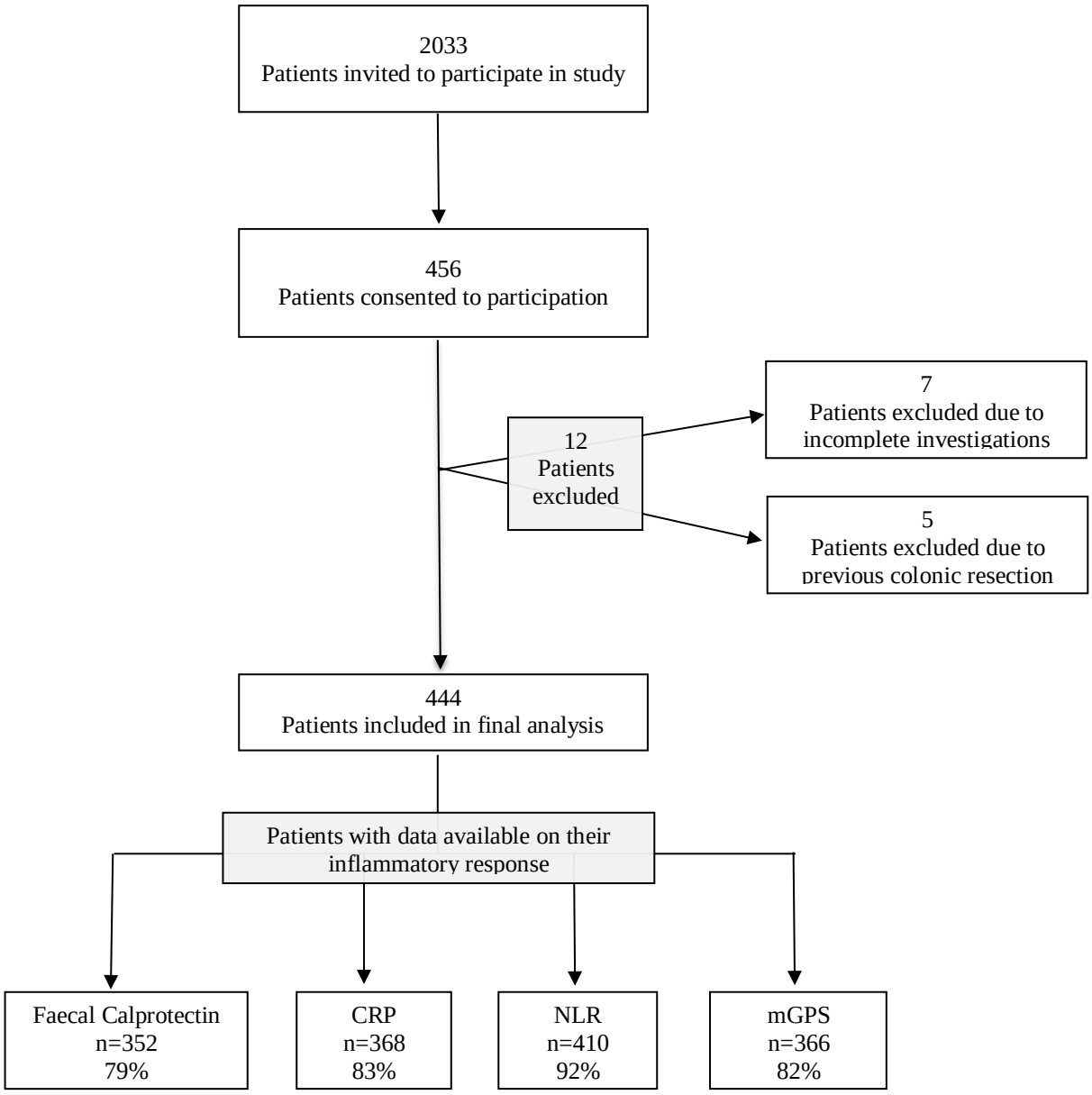

Figure 5.1. Flowchart of patient recruitment

6.0 The relationship between faecal calprotectin, clinicopathological characteristics, and systemic inflammation in colorectal cancer?

6.1 Introduction

The relationship between cancer and inflammation is well recognised. [173] CRC, the role of both colonic inflammation, as well as systemic inflammation can help determine cancer progression and survival. [193, 147, 194]

Calprotectin detected in the faeces is a sensitive measure of colonic inflammation. [206] It is found predominantly in the cytoplasm of neutrophils and the membrane of monocytes and is released upon cell death or damage. [210, 206-208] Calprotectin enters the bowel lumen by migration and is resistant to enzymatic degradation therefore can be readily detected in faeces [213, 207, 209, 211].

In chapter 3, I performed a systematic review of FC and colorectal neoplasia to characterise the relationship FC has with CRC. In a total of 35 studies, I found that CRC patients are more likely than controls to have an elevated FC OR 5.19, 95% CI 3.12-8.62, P<0.001. However there was a lack of evidence to show a relationship between lesser degrees of neoplasia i.e. adenomas, with FC. Although FC is not specific to CRC, to allow it to be used as a screening or diagnostic tool, it does confirm a correlation with FC and CRC, and the relationship between CRC and inflammation.

This was confirmed in chapter 4. In a FOBT positive screening population, FC was strongly associated with CRC (sensitivity 92.8%, specificity 41.7% for CRC, at 50µg/g). CRC patients had a higher median FC (138.5ug/g, p <0.05), in comparison to those without CRC, and 13/14 had a FC >50µg/g (93%).

As noted in the previous chapter, it is not clear whether FC acts as a surrogate marker of the overall inflammatory tumour microenvironment or as a marker of specific components. Can FC therefore be used as an adjunct in CRC staging? The majority of CRCs are diagnosed at an early, and potentially curative stage. [287] However many of these cancers will later recur, some at an early stage.[288, 289] One focus of research in recent years has been to determine why this happens and how to identify these patients early to improve the recurrence rate. The local inflammatory response in the tumour microenvironment is known to dictate disease progression and survival and is one such area that may be targetable for modification, possibly at a neoadjuvant stage. [193, 290, 164] Present methods of assessing the tumour microenvironment rely on tissue sampling, and are typically reliant on post-operative resection specimens. [166] Despite the acknowledged prognostic relationship, assessment of the local inflammatory response is currently not a requirement of in staging of CRC. This may be due to the heterogeneity of the different scores, the technical skills required to assess the LIR and the potential for assessor variability in scoring.

As stated in chapter 3, there is less literature on FC's correlation with CRC stage, with only seven papers reporting on this. Only one study reported T-stage significantly (p=0.022) correlated with FC. [246] Two further studies showed a non-significant correlation. [247, 211] Considering size of tumour rather than depth (i.e. T-stage), there was only one study, and there was no correlation reported. [211] If FC does correlate with the tumour microenvironment it would be advantageous for CRC staging. It could allow an easy assessment of the LIR in CRC at an earlier time in cancer staging. This could potentially help to dictate neoadjuvant treatment based upon those thought to be at high risk of recurrence.

In inflammatory bowel disease it has been shown that disease activity, FC and histology all correlate. [224] Does the same follow if the disease is cancer? Kristinsson et al reported no significant difference in FC between histological grading's. [211] In 2014 Lehman et al carried out the first study assessing correlation of FC with tumour and histopathological parameters of local inflammation in colorectal cancer. This showed no significant correlation between FC and markers of local inflammation, nor with tumour histology. [246] More evidence is needed to assess if FC correlates with the tumour microenvironment or tumour histology.

Systemic inflammation is a predictor of poorer outcome in both early resectable colorectal cancer as well as advanced disease. [196, 291] The relationship between systemic inflammation and intraluminal colonic inflammation (measured using FC) is unclear. In chapter 5 I analysed FCs association with markers of SIR in a bowel screening FOBT positive cohort. In this cohort there was no significant relationship between FC and markers of SIR. Kristinsson et al. found no significant correlation in colorectal cancer between FC and markers of systemic inflammation (CRP, CEA, plasma calprotectin). [249] However given the minimal literature on this topic it would be interesting to further analyse whether there is a correlation with FC and SIR, in CRC patients.

Although FC value as a diagnostic tool is poor, I hypothesise that FC may be a simple measure of local colonic inflammation in the tumour microenvironment in CRC and therefore be of value as an adjunct in CRC staging. In this chapter I aim to analyse FC correlation with tumour histology, microenvironment and inflammation. The primary study objective is to analyse this relationship between FC and tumour histology. The secondary objective was to analyse the relationship between FC and systemic inflammation, in CRC.

6.2 Methods

6.2.1 Patients

'Investigation of the local and systemic inflammatory response and of dietary habits in those attending for investigation via the National Health Service Colorectal Cancer Screening Programme', was the study set up to examine multiple research questions about patients attending for colonoscopy via SBSP. This population was examined in chapters 4 and 5, with the full methodology being found in chapter 4. Patients with colorectal cancer identified in this study were extracted to be studied as a separate cancer specific cohort.

Due to the small number of patients with colorectal cancer, these patients were combined with data collected in earlier years, to increase the number of patients analysed. From 2011 to 2014 CRC patients in Glasgow Royal Infirmary were identified at the colorectal MDT. Patients were contacted and asked to provide a FC sample for analysis, prior to starting any oncological or surgical treatment.

CRC patients without a valid FC were excluded from this study.

6.2.2 Methods

As in chapters 4 and 5, consenting patients from the SBSP study were instructed to provide a stool sample for calprotectin estimation. This was to be taken as close as possible to their colonoscopy date, but prior to commencement of bowel preparation. Patients then returned the sample on the day of their colonoscopy. Patients identified between 2011 and 2014, were contacted and provided a stool sample for calprotectin following their CRC diagnosis. Faecal calprotectin, for both cohorts, was analysed by the NHS biochemistry laboratory, at Glasgow Royal Infirmary using standard clinical grade assays (Bühlmann fCAL® ELISA). As discussed in chapter 3, local guidelines and reference ranges created three groupings for analysis: <50µg/g, 50-200µg/g and >200 µg/g.

For both patient groups, patient demographics were recorded in a prospectively maintained database on an encrypted hard drive. Using the patient's digital records, data including smoking status, drug use, BMI, blood results for markers of systemic inflammation, FC and tumour histology were obtained. Both databases were combined for analysis.

The same markers and cut-offs for SIR were used as discussed in chapter 5. Tumour histology displayed as per local histology reporting. Tumour size was split into two groups for analysis, using the median value.

6.2.3 Objectives

The primary objective was to analyse the relationship between FC and tumour histology. The secondary objective was to analyse the relationship between FC and SIR in CRC.

6.2.4 Statistical Analysis

Categorical data regarding patient characteristics were compared using the Chi square test and Chi square test for linear association where appropriate. In addition, faecal calprotectin results were expressed as medians, and analysed using Kruskal-Wallis. A p value of <0.05 was considered significant for all analysis. Statistical analysis was performed using SPSS version 25.0 for Windows (IBM Corporation, Armonk, NY).

6.2.5 Ethics

The SBSP study was approved by the NHS Greater Glasgow and Clyde Research and Development department, and the Research Ethics Service (REC number: 15/WA/0053).

6.3 Results

6.3.1 Patient characteristics

Fifty-six patients (thirty-eight from the first cohort, and eighteen from the second cohort) were included in the final analysis. Table 6.1 describes the demographics of the study participants. 41% (n=23) were ≥ 70 years old. The majority were male (64%, n=36). 72% (n=37) had a BMI <30. 56% (n=30) had never smoked. All patients underwent elective colorectal resection No patient had emergency surgery. 64% (n=30) were American Society of Anaesthesiologists (ASA) classification 2, indicating mild systemic disease. 41% (n=22) had a stage III colorectal cancer. 84% (n=47) had an elevated FC ≥50µg/g, and 50% (n=28) had a FC ≥200 µg/g. 9% (n=5) had an elevated mGPS (1-2), whereas 57% (n=31) had an elevated CRP ≥3.

6.3.2 Patient characteristics and faecal calprotectin

The relationship between patient characteristics is displayed in Table 6.1. Male patients had a higher median FC (248.5µg/g vs. 123.5µg/g) and more patients with a FC≥200 (56% vs. 40%), in comparison to females. Patients in the most deprived SIMD quintiles have the highest median FC and higher percentage of patients with FC≥200µg/g, compared to SIMD quintiles 3-5. Patients with a BMI 35+ have the highest percentage (80%) of patients with FC≥200µg/g, and a median FC of 229µg/g.

6.3.3 Tumour factors and faecal calprotectin

Table 6.2 shows the relationship between tumour histology and faecal calprotectin. Patients with T4 tumours had the highest median FC (321µg/g), with 67% having a FC ≥200µg/g. 29% of those with T1 tumours had a FC ≥200µg/g. Patients with nodal or metastatic disease had higher median FC, compared to those without.

Patients with a tumour ≥3.5cm had a higher median FC 251.5µg/g, and 67% had a FC ≥200µg/g, in comparison to those with a tumour <3.5cm (median 164 µg/g, and 48% FC ≥200µg/g).

Patients with rectal cancer had a slightly higher median FC compared to colon cancer (219µg/g vs 169µg/g), but no significant correlation.

Patients with peritoneal involvement had significantly higher median FC, compared to those without - median FC (405µg/g vs 164µg/g), $p < 0.05$. 89% of patients with peritoneal involvement had FC ≥200µg/g, compared to 44% in those without peritoneal involvement ($p<0.05$). Poorly differentiated tumours had a higher median FC (281.5µg/g) than well/moderate differentiated tumours (169µg/g), but not significantly. Patients with margin involvement and tumour perforation had higher median FC, however there were only two patients with these positive findings. No other histological factor, including mucin, venous invasion or perineural invasion showed a significant relationship with FC.

I assessed the same relationship between tumour histology and faecal calprotectin, but excluding rectal cancer patients. This does not change the relationships described above, with higher stage and larger tumours having higher FC. Peritoneal involvement remains a significant relationship, with a higher median FC (405µg/g), $p<0.05$ and higher percentage (86%) with FC ≥200 µg/g, $p<0.05$.

6.3.4 Tumour factors and systemic inflammatory response

The relationship between tumour histology and the SIR (CRP) is displayed in Table 6.3a. Patients with a tumour ≥3.5cm, had a higher median CRP 5.0, compared to 2.5 in those with tumour <3.5cm. Patients with stage IV or metastatic cancer had a higher median CRP 6.2, with median CRP 3.1 in those without metastatic disease. Patients without perineural or venous invasion had higher percentage patients with a CRP≥3, than those with invasion ($p<0.05$). No other histological factor showed a significant relationship with CRP.

The relationship between NLR and tumour histology is displayed in Table 6.3b. Patients with a tumour ≥ 3.5cm, had a higher median NLR 2.8, compared to 2.2 in those with tumour <3.5cm. ($p<0.05$). Patients with T1/2 disease were had a higher percentage of patients with an NLR<3, in comparison to those with T3/4 disease.

6.3.5 Faecal calprotectin and systemic inflammatory response

Table 6.4 displays the relationship between FC and SIR illustrated by inflammation based prognostic scores. Patients have a higher mGPS, CRP, NLR, WCC, neutrophils, monocytes and basophils, all have higher median FC, but not significantly. 80% (n=4) of patients with an elevated mGPS (1-2) had a FC ≥200µg/g, with a median FC 251µg/g, compared to 169µg/g in patients with an mGPS 0. Patients with a CRP ≥3 had median FC 229µg/g, compared to 164µg/g in patients with CRP<3. 100% (n=2) of patients with an elevated WCC >11 had a FC ≥200µg/g, with a higher median FC 1359µg/g, compared to 158µg/g in patients with WCC <8.5. 100% (n=3) of patients with an elevated neutrophils ≥7.5 had a FC ≥200µg/g, with a higher median FC 948µg/g, compared to 164µg/g in patients with neutrophils <7.5. 80% (n=4) of patients with an elevated monocytes >0.9 had a FC ≥200µg/g, with a higher median FC 501µg/g, compared to 164µg/g in patients with monocytes ≤0.9.

6.3.6 Overall inflammation and cancer stage

As in chapter 5, groupings of overall inflammation have been created by combining markers of SIR (CRP/NLR) with markers of LIR (FC). Table 6.5 displays the four groups; 1: low LIR and SIR, 2: low LIR and high SIR, 3: high LIR and low SIR, and 4: high LIR and SIR.

Table's 6.6a/b display overall inflammation with CRP used as the marker of SIR. In CRC stage I/II 23% (n=7) patients, and 21% (n=5) of CRC stage III/IV have an inflammatory score of 1. More

patients with stage III/IV CRC have inflammatory score 4 in comparison to stage I/II (38%, n=9 vs. 27%, n=8) (p=0.305). In T1-2, 25% (n=4) have an inflammatory score of 1, and in T4 18% (n=2). In T1-2 tumours 13% (n=2) have an inflammatory score of 4, compared to 42% (n=10) in T3 and 45% (n=5) in T4 (p=0.084).

Table's 6.6c/d display overall inflammation with NLR used as the marker of SIR. In CRC stage I/II 42% (n=13) patients, and 32% (n=8) of CRC stage III/IV have an inflammatory score of 1. More patients with stage III/IV CRC have inflammatory score 4 in comparison to stage I/II (20%, n=5 vs. 13%, (n=4) (p=0.252). In T1-2, 59% (n=10) have an inflammatory score of 1, and in T4 0%. In T1-2 tumours 6% (n=1) have an inflammatory score of 4, compared to 25% (n=6) in T3 and 17% (n=2) in T4 ($p<0.05$).

6.4 Discussion

In this chapter I examined the relationship between FC and tumour histology as well as the relationship between FC and systemic inflammation, in CRC. In general there is a non-significant trend towards larger more advanced tumours having higher levels of FC.

There are many thoughts as to why FC is elevated in CRC; Elevated calprotectin may occur as a direct result of tumour inflammation as it has been shown that the expression of calprotectin correlates with the degree of neutrophilic infiltration. [292] It has also been reported that calprotectin may play a role in inhibiting tumour cell lines by inducing apoptosis, as studies have shown that calprotectin inhibits the growth of several human cell lines. [211, 212] It has been postulated that polymorph leukocytes are activated by cancer, acting as killer cells or that neutrophils are recruited to the tumour secondary to the local production of chemotactic factors, both resulting in calprotectin release from cell death. [211, 213] Many studies have proven that FC significantly reduces following resectional surgery. [211, 246, 293] While this again is evidence of high FC levels in CRC it does not confirm correlation with levels of disease activity or stage.

In ulcerative colitis, calprotectin levels are proportional to mucosal neutrophilic infiltration. Similarly, it has been found that neutrophils infiltrate neoplastic tissue as per the volume of the neoplasm. [213, 294] It would thereby follow that a larger intraluminal tumour burden should have higher levels of inflammation i.e. FC. In this analysis 56 patients with colorectal cancer, and pre-operative FC results, were studied. Patients with T4 CRC had the highest median FC of all T-stages, and the highest proportion with a FC ≥200µg/g. Patients with nodal or metastatic disease had higher median FC, compared to those without. Overall higher stage patients had a higher median FC, and were more likely to have a FC ≥200µg/g, however not significantly. Lehman et al reported similar increased FC in higher stage CRCs, and Kristinsson et al reported slightly lower FC levels in Dukes' A CRCs but not significantly. [211, 246] In the current cohort, patients with larger tumours (≥3.5cm) had a higher median FC and were more likely to have a FC ≥200µg/g, in comparison to those with a tumour <3.5cm, however again not significantly. Kristinsson and Limberg reported no difference in FC between tumour sizes. [248, 211] The small number of patients studied may explain this variation, and the overriding relationship between FC and tumour size is not yet established. Further studies are required to prove a relationship between tumour size and FC. If tumour size correlates with FC, the size in which the tumour starts to cause increasing FC is yet to be determined.

In this cohort patients with rectal cancer had a slightly higher median FC compared to colon cancer (219µg/g vs 169µg/g), but no significant correlation between location and FC was seen. Kristinsson et al 2001 also reported slightly higher levels of FC in rectal cancer vs colonic, but not significantly. [211, 249] While Tibble et al reported no difference in FC levels in colon and rectum. Limburg et al reported that proximal CRCs had a significantly higher FC than distal CRCs (or both

proximal and distal tumours). [248] No other studies reported a significant difference in FC with tumour location. [261, 249, 246, 271] There are a number of ideas regarding how to better understand the relationship between FC and tumour location; it may be that more proximal colonic tumours have the FC concentration diluted as it travels through the remainder of the colon resulting in lower FC levels. However this would be confounded by all the other potential factors altering FC levels, resulting in the variability and discrepancies. CRC is also well known to display considerable heterogeneity, with variations in morphology, genetics as well as the TME. Different areas of the colon show different levels of immunological markers even in the healthy colon, with higher levels of inflammatory cells in the proximal colon. [295] CRC which has developed in the proximal colon would be exposed to increased immune activity, with MSI positive tumours (with presence of tumour infiltrating lymphocytes (TILs), TILs themselves more commonly in the proximal colon. [95] The presence and density of TILs in CRC is associated with a better prognosis. [166] If FC is a marker for specific components of the TME, this may explain the variation.

Patients in this study with peritoneal involvement had significantly higher median FC, compared to those without, and significantly higher likelihood of having a FC ≥200µg/g. No other histological factor showed a significant relationship with FC. Poorly differentiated tumours did have a higher median FC than well/moderate differentiated, but not significantly and patients with margin involvement and tumour perforation had higher median FC, however there were only two patients with these positive findings. The few other studies that have looked at correlation between FC and tumour histology have not shown any significant correlation. [246, 211]

In CRC the relationship with local and systemic inflammation is well known, if FC is potentially a marker of local inflammation is there a connection between FC and SIR. Given the opposite prognostic values of a high LIR and high SIR, in CRC, it may be hypothesised that high FC levels would not correlate with a high SIR. Patients in this cohort with a higher mGPS, CRP, NLR, WCC, neutrophils, monocytes and basophils, all have higher median FC, but not significantly. The majority of patients with an elevated mGPS had a FC ≥200µg/g, as was the case with WCC, neutrophils, monocytes but again not significantly. This suggests that there could potentially be a correlation between elevated FC and elevated markers of the SIR in CRC. Kristinsson et al reported high FC in CRC, but no significant correlations among CRP, CEA, and faecal or plasma calprotectin. [249] Is FC a reflection of CRC and more advanced disease, but not representative of the local inflammatory response?

This chapter represents one of few studies on faecal calprotectin's correlation with tumour histology and SIR in CRC. This novelty, in conjunction with the advancing area of neoadjuvant treatment in CRC is a strength of this work. Unfortunately the small number of patients included in this study precludes definitive conclusions being drawn, however provides a good basis for the

development of future studies. Another limiting factor is the lack of information regarding the earlier cohort of patients in whom FC was collected. While there are potential non-significant trends observed between FC, tumour histology and inflammation in this study, it would be interesting to see if these results are amplified when study numbers are increased.

If FC is shown to be significantly elevated in more advanced CRC it could be used as an adjunct to staging, and potentially as an indicator of what patients may benefit from adjuvant and neoadjuvant therapy. This is particularly relevant given that neoadjuvant chemotherapy in colon cancer has been found to be safe and effective. [125]

It would therefore be of interest to scale up this study and also include more specific information particularly on tumour location. It would also be of interest to assess the tumour microenvironment assessing what components of the LIR that FC may correlate with, and be a marker of. Finally patient outcome data including survival and recurrence rates would be of interest in assessing FC usefulness in staging and determining treatment.

In conclusion, this chapter has shown that there may be a potential relationship between higher levels of FC and colorectal cancers with larger, more advanced tumours, displaying negative prognostic factors but more work is required to clarify this.

6.5 Table and Figures

Table 6.1 Faecal calprotectin and patient factors

	n (%)	Median FC (IQR)	P-value	FC <200 n (%)	FC ≥200 n (%)	P-value*
All patients	56	194.0 (88.5-371.5)	-	28 (50)	28 (50)	-
Age (years)						
<60	12 (21)	255.5 (151.3-386.3)	0.043	4 (33)	8 (67)	0.863
60-69	21 (38)	113.0 (76.5-251.5)		15 (71)	6 (29)	
70+	23 (41)	249.0 (98.0-405)		9 (39)	14 (61)	
Sex						
Female	20 (36)	123.5 (85.5-315.0)	0.403	12 (60)	8 (40)	0.265
Male	36 (64)	248.5 (91.8-401.5)		16 (44)	20 (56)	
SIMD Quintile						
1-3	24 (43)	238.5 (90.5-397.8)	0.667	10 (42)	14 (58)	0.280
4-5	32 (57)	160.0 (85.5-352.5)		18 (56)	14 (44)	
BMI (52)[a]						
<25	18 (35)	249.5 (98.8-390.0)	0.221	7 (39)	11 (61)	0.991
25-29.9	19 (37)	113.0 (52.0-315.0)		13 (69)	6 (32)	
30-34.9	10 (19)	113.0 (87.0-315.8)		6 (60)	4 (40)	
35+	5 (10)	229.0 (158.5-1009.5)		1 (20)	4 (80)	
Smoking (54)[a]						
Never	30 (56)	233.5 (70.0-411.8)	0.784	14 (47)	16 (53)	0.584
Ever	24 (44)	166.5 (93.3-272.0)		13 (54)	11 (46)	
ASA (47)[a]						
1-2	36 (77)	166.5 (78.75-360.75)	0.269	19 (53)	17 (47)	0.341
3-4	11 (23)	249.0 (206.0-432.0)		4 (36)	7 (67)	

Table 6.1. Faecal calprotectin and patient factors

SIMD: Scottish Index of Multiple Deprivation BMI: body mass index NSAIDS: non-steroidal anti-inflammatory drugs, PPI: proton pump inhibitors ACE: angiotensin converting enzyme inhibitor, ASA: American Society of Anaesthesiologists classification, CRP: c-reactive protein, NLR: neutrophil lymphocyte ratio, CRC: colorectal cancer

[a] Number of patients when incomplete data available *chi squared

Table 6.2 Faecal calprotectin and tumour factors

	n (%)	Median FC (IQR)	P-value	FC <200 n (%)	FC ≥200 n (%)	P-value*
All patients	56	194.0 (88.5-371.5)	-	28 (50)	28 (50)	-
Site						
Colon	31 (55)	169.0 (98.0-376.0)	1.000	16 (52)	15 (48)	0.788
Rectum	25 (45)	219.0 (80.0-384.0)		12 (48)	13 (52)	
Tumour Size (47)[a]						
<3.5cm	23 (49)	164.0 (52.0-336.0)	0.659	12 (52)	11 (48)	0.196
≥3.5cm	24 (51)	251.5 (149.3-425.3)		8 (33)	16 (67)	
T Stage (53)[a]						
1	7 (13)	121.0 (29.0-376.0)	0.391	5 (71)	2 (29)	0.067
2	10 (19)	138.0 (89.5-326.0)		6 (60)	4 (40)	
3	24 (45)	238.5 (100.0-330.8)		10 (42)	14 (58)	
4	12 (23)	321.0 (136.3-483.8)		4 (33)	8 (67)	
N Stage						
0	33 (59)	164.0 (80.5-347.0)	0.400	19 (58)	14 (42)	0.174
1-2	23 (41)	249.0 (98.0-405.0)		9 (39)	14 (61)	
Metastatic Disease						
No	51 (91)	169.0 (88.0-358.0)	1.000	26 (51)	25 (49)	0.639
Yes	5 (9)	263.0 (72.5-613.0)		2 (40)	3 (60)	
Stage						
I	17	120 (53.0-267.0)		12 (71)	5 (29)	
II	14	256.5 (131.3-469.0)		6 (43)	8 (57)	0.080
III	20	248.5 (92.0-370.5)	0.267	8 (40)	12 (60)	
IV	5	263.0 (72.5-613.0)		2 (40)	3 (60)	
Differentiation (55)[a]						
Well-mod	45 (82)	169.0 (87.0-347.0)	0.679	23 (51)	22 (49)	0.525
Poor	10 (18)	281.5 (95.5-591.0)		4 (40)	6 (60)	
Mucin (53)[a]						
No	46 (87)	238.5 (89.5-393.0)	0.957	22 (48)	24 (52)	0.806
Yes	7 (13)	219.0 (77.0-251.0)		3 (43)	4 (57)	
Venous Invasion (53)[a]						
No	18 (34)	246.0 (66.8-394.3)	0.848	8 (44)	10 (56)	0.776
Yes	35 (66)	219.0 (90.0-376.0)		17 (49)	18 (51)	
Perineural Invasion (53)[a]						
No	49 (92)	229.0 (89.0-397.0)	0.631	23 (47)	26 (53)	0.906
Yes	4 (8)	189.5 (53.5-299.3)		2 (50)	2 (50)	
Margin Involvement (52)[a]						
No	50 (96)	224.0 (89.5-379.3)	0.471	24 (48)	26 (52)	0.182
Yes	2 (4)	1042.5 (315.0- -)		0 (0)	2 (100)	
Peritoneal Involvement (54)[a]						
No	45 (83)	164.0 (76.5-300.5)	0.028	25 (56)	20 (44)	0.015
Yes	9 (17)	405.0 (267.5-562.5)		1 (11)	8 (89)	
Tumour Perforation (54)[a]						
No	52 (96)	224.0 (88.5-371.5)	0.471	25 (48)	27 (52)	0.957
Yes	2 (4)	899.5 (29.0- -)		1 (50)	1 (50)	

Table 6.2. Faecal calprotectin and tumour factors

[a] Number of patients when incomplete data available *chi squared

Table 6.3a SIR and tumour factors - CRP

	n (%)	Median CRP (IQR)	P-value	CRP <3 n (%)	CRP ≥3 n (%)	P-value*
All patients	54	3.55	-	23 (43)	31 (57)	-
Site						
Colon	31 (57)	3.70 (1.1-6.9)	1.000	15 (48)	16 (52)	0.317
Rectum	23 (43)	3.4 (1.3-6.2)		8 (35)	15 (65)	
Tumour Size (46)[a]						
<3.5cm	22 (48)	2.5 (1.0-4.7)	0.376	11 (50)	11 (50)	0.571
≥3.5cm	24 (52)	5.0 (1.2-7.7)		10 (42)	14 (58)	
T Stage (51)[a]						
1	6 (12)	5.6 (0.93-8.1)	0.768	2 (33)	4 (67)	0.938
2	10 (20)	1.7 (1.0-5.7)		6 (60)	4 (40)	
3	24 (47)	3.6 (1.3-6.9)		9 (38)	15 (63)	
4	11 (22)	3.0 (1.2-5.2)		5 (46)	6 (55)	
N Stage						
0	32 (59)	4.7 (1.4-6.2)	0.374	12 (38)	20 (63)	0.361
1-2	22 (41)	2.6 (1.0-6.8)		11 (50)	11 (50)	
Metastatic Disease						
No	49 (91)	3.1 (1.1-6.0)	0.348	22 (45)	27 (55)	0.283
Yes	5 (9)	6.2 (3.5-28.0)		1 (20)	4 (80)	
Stage						
I	16	3.0 (1.0-6.8)		8 (50)	8 (50)	
II	14	4.7 (2.0-6.0)	0.128	4 (29)	10 (71)	0.669
III	19	2.2 (1.0-5.1)		10 (53)	9 (47)	
IV	5	6.2 (3.5-28.0)		1 (20)	4 (80)	
Differentiation						
Well-mod	44 (81)	3.8 (1.2-6.6)	0.726	18 (41)	26 (59)	0.600
Poor	10 (19)	2.7 (1.1-7.3)		5 (50)	5 (50)	
Mucin (52)[a]						
No	45 (87)	3.7 (1.3-6.0)	1.000	18 (40)	27 (60)	0.393
Yes	7 (14)	2.2 (0.9-7.8)		4 (57)	3 (43)	
Venous Invasion (52)[a]						
No	18 (35)	5.0 (2.8-6.9)	0.145	4 (22)	14 (78)	0.033
Yes	34 (65)	2.1 (1.1-5.6)		18 (53)	16 (57)	
Perineural Invasion (52)[a]						
No	48 (92)	4.2 (1.4-6.6)	0.118	18 (38)	30 (63)	0.015
Yes	4 (8)	0.7 (0.4-0.8)		4 (100)	0 (0)	
Margin Involvement (50)[a]						
No	48 (96)	3.3 (1.2-6.2)	0.470	21 (44)	27 (56)	0.861
Yes	2 (4)	17.3 (0.5--)		1 (50)	1 (50)	
Peritoneal Involvement (52)[a]						
No	43 (83)	3.7 (1.2-6.2)	1.000	18 (42)	25 (58)	0.887
Yes	9 (17)	3.0(1.1-8.6)		4 (44)	5 (56)	
Tumour Perforation (52)[a]						
No	50 (96)	3.3 (1.2-6.1)	0.471	22 (44)	28 (56)	0.217
Yes	2 (4)	19.5 (5.0--)		0 (0)	2 (100)	

Table 6.3a. SIR and tumour factors – CRP

[a] Number of patients when incomplete data available *chi squared

Table 6.3b SIR and tumour factors - NLR

	n (%)	Median NLR (IQR)	P-value	NLR <3 n (%)	NLR ≥3 n (%)	P-value*
All patients	56		-	40 (71)	16 (29)	-
Site						
Colon	31 (55)	2.5 (2.1-4.3)	0.291	20 (65)	11 (36)	0.202
Rectum	25 (45)	2.3 (2.0-2.9)		20 (80)	5 (20)	
Tumour Size (47)[a]						
<3.5cm	23 (49)	2.2 (1.8-2.9)	0.028	18 (78)	5 (22)	0.143
≥3.5cm	24 (51)	2.8 (2.3-4.4)		14 (58)	10 (42)	
T Stage (53)[a]						
1	7 (13)	2.5 (1.7-4.2)	0.108	5 (71)	2 (29)	
2	10 (24)	2.6 (1.6-2.8)		10 (100)	0 (0)	0.078
3	24 (45)	2.3 (1.9-3.4)		16 (67)	8 (33)	
4	12 (23)	3.0 (2.4-5.6)		6 (50)	6 (50)	
N Stage						
0	33 (59)	2.5 (2.0-3.7)	0.752	23 (70)	10 (30)	0.731
1-2	23 (41)	2.4 (2.0-3.0)		17 (74)	6 (26)	
Metastatic Disease						
No	51 (91)	2.5 (1.9-3.2)	0.646	37 (73)	14 (28)	0.553
Yes	5 (9)	2.4 (2.3-3.7)		3 (60)	2 (40)	
Stage						
I	17 (30)	2.5 (1.6-2.7)	0.578	15 (88)	2 (12)	
II	14 (25)	3.0 (2.0-4.6)		7 (50)	7 (50)	0.327
III	20 (36)	2.4 (1.9-3.3)		15 (75)	5 (25)	
IV	5 (9)	2.4 (2.3-3.7)		3 (60)	2 (40)	
Differentiation (55)[a]						
Well-mod	45 (82)	2.4 (2.0-2.8)	0.226	35 (78)	10 (22)	0.074
Poor	10 (18)	3.0 (2.0-3.7)		5 (50)	5 (50)	
Mucin (53)[a]						
No	46 (87)	2.5 (2.1-3.3)	0.072	32 (70)	14 (30)	0.377
Yes	7 (13)	1.8 (1.6-2.7)		6 (86)	1 (14)	
Venous Invasion (53)[a]						
No	18 (34)	2.2 (1.6-4.3)	0.248	13 (72)	5 (28)	0.952
Yes	35 (66)	2.5 (2.2-3.1)		25 (71)	10 (29)	
Perineural Invasion (53)[a]						
No	49 (93)	2.4 (2.0-3.0)	0.674	36 (74)	13 (27)	0.316
Yes	4 (8)	2.8 (1.8-9.3)		2 (50)	2 (50)	
Margin Involvement (52)[a]						
No	50 (96)	2.5 (2.0-3.4)	0.536	35 (70)	15 (30)	0.548
Yes	2 (4)	3.0 (2.5- -)		1 (50)	1 (50)	
Peritoneal Involvement (54)[a]						
No	45 (83)	2.4 (1.9-3.2)	0.150	32 (71)	13 (29)	0.790
Yes	9 (17)	2.7 (2.2-5.6)		6 (67)	3 (33)	
Tumour Perforation (54)[a]						
No	52 (96)	2.4 (1.9-3.2)	0.252	37 (71)	15 (29)	0.520
Yes	2 (4)	4.5 (2.3- -)		1 (50)	1 (50)	

Table 6.3b. SIR and tumour factors – NLR

[a] Number of patients when incomplete data available *chi squared

Table 6.4 Faecal calprotectin and systemic inflammation based prognostic scores in CRC						
	n (%)	Median FC (IQR)	P-value	FC <200 n (%)	FC ≥200 n (%)	P-value*
All patients	56	194.0 (88.5-371.5)	-	28 (50)	28 (50)	-
mGPS (54)[a]						
0	49 (91)	169.0 (94.0-367.0)	0.369	25 (51)	24 (49)	0.186
1-2	5 (9)	251.0 (158.5-1247.5)		1 (20)	4 (80)	
CRP (54)[a]						
<3	23 (43)	164.0 (98.0-376.0)	1.000	12 (52)	11 (48)	0.610
≥3	31 (57)	229.0 (88.0-389.0)		14 (45)	17 (55)	
NLR						
<3	40 (71)	160.0 (85.0-352.5)	0.767	21 (53)	19 (48)	0.554
≥3	16 (29)	246.0 (132.0-397.8)		7 (44)	9 (56)	
NLR						
<5	50 (89)	191.5 (87.0-341.5)	0.624	25 (50)	25 (50)	1.000
≥5	6 (11)	272.5 (102.5-411.8)		3 (50)	3 (50)	

Table 6.4. Faecal calprotectin and systemic inflammation based prognostic scores in CRC

[a] Number of patients when incomplete data available *chi squared

Table 6.5 Overall inflammation coding

1	Low LIR/ Low SIR
2	Low LIR/ High SIR
3	High LIR/ Low SIR
4	High LIR/ High SIR

Table 6.5. Overall Inflammation Coding
Low LIR: FC<200
High LIR: FC ≥200
Low SIR: CRP/NLR<3
High SIR: CRP/NLR ≥3

Table 6.6a Inflammation and CRC stage - CRP

	n (%)	I/II n (%)	III/IV n (%)	P-value
1	12 (22)	7 (23)	5 (21)	
2	14 (26)	10 (33)	4 (17)	0.305
3	11 (20)	5 (17)	6 (25)	
4	17 (32)	8 (27)	9 (38)	

Table 6.6a. Inflammation and CRC stage - CRP

Table 6.6b Inflammation and CRC T-stage - CRP

	n (%)	T1-2 n (%)	T3 n (%)	T4 n (%)	P-value
1	11 (22)	4 (25)	5 (21)	2 (18)	
2	12 (24)	6 (38)	5 (21)	1 (9)	0.084
3	11 (22)	4 (25)	4 (17)	3 (27)	
4	17 (33)	2 (13)	10 (42)	5 (45)	

Table 6.6b. Inflammation and CRC T-stage - CRP

Table 6.6c Inflammation and CRC stage - NLR

	n (%)	I/II n (%)	III/IV n (%)	P-value
1	21 (38)	13 (42)	8 (32)	
2	7 (13)	5 (16)	2 (8)	0.252
3	19 (34)	9 (29)	10 (40)	
4	9 (16)	4 (13)	5 (20)	

Table 6.6c. Inflammation and CRC stage - NLR

Table 6.6d Inflammation and CRC T-stage - NLR

	n (%)	T1-2 n (%)	T3 n (%)	T4 n (%)	P-value
1	18 (34)	10 (59)	8 (33)	0 (0)	
2	7 (13)	1 (6)	2 (8)	4 (33)	0.014
3	19 (36)	5 (29)	8 (33)	6 (50)	
4	9 (17)	1 (6)	6 (25)	2 (17)	

Table 6.6d. Inflammation and CRC T-stage - NLR

7.0 Faecal calprotectin and the local inflammatory response in colorectal cancer, a pilot study

7.1 Introduction

The staging of patients with colorectal cancer is currently based on the TNM classification. However, it is recognised that TNM staging does not adequately predict true recurrence risk for all patients. The majority of CRCs are diagnosed at an early, potentially curative stage. [287] Many cancers recur, some at an early stage. [288, 289] A multitude of factors are thought to play into recurrence risk and survival in CRC including inflammation and components of the TME.

Inflammation, both local and systemic, are now recognised to play an important role in the prognosis and survival of colorectal cancer. In particular, the local inflammatory response in the TME is known to dictate disease progression. Itis a key area that may be targetable for modification, possibly in the neoadjuvant or adjuvant settings. [193, 290, 164]

Measurement of the local inflammatory response (LIR) in the TME includes the Klintrup-Makinen grade (KM grade) and tumour stroma percentage (TSP). A high KM grade is an independent prognostic factor for improved cancer specific survival in CRC. [162, 159, 163] The presence of a higher stromal to epithelial volume within the tumour, has been shown to be a stage independent marker of reduced survival in patients with operable CRC. [146-149] More information on KM and TSP can be found in sections 1.6.4.2 and 1.6.2.7.

The adaptive immune system is mediated by the T-cell lymphocytes and B-cell antigen receptors. [175] In a cell-mediated immune response T cells react directly against the foreign antigen. [177] Tumour-infiltrating lymphocytes (TILs) are an important prognostic feature of CRC, being significantly associated with cancer specific and overall survival, with high levels of generalized TILs having improved overall survival. [201] Specific immune cell types including subsets of T-lymphocytes: CD3+, CD8+ cytotoxic, CD45RO+ memory, FOXP3+, tumour associated macrophages (TAMs), relate to recurrence and survival. [202, 193] The Immunoscore is a scoring system which assesses the density of T-cell lymphocytes within the tumour. A high Immunoscore is associated with a reduced risk of recurrence, disease-free survival and overall survival. [164, 166-168] More information on the local inflammatory response and inflammatory scores can be found in sections 1.6.4 and 1.7.4.

Present methods of assessing the tumour microenvironment rely on tissue sampling and on post-operative specimens. They have not yet translated into use in clinical practice. [166]

FC, a measure of colonic inflammation, represents another assessment of inflammation. I have shown in a review of the current literature (chapter 3) and in analysis of local data (chapter 4) that

CRC is strongly associated with elevated FC levels, and that larger, more advanced tumours were more likely to have higher levels of FC (chapter 6).

Lehman et al carried out a study of 80 patients in 2014 assessing correlation of FC with tumour and histopathological parameters of local inflammation in colorectal cancer. This showed no significant correlation between FC and markers of local inflammation (KM grade, MPO, CD45R0, TIA-1, CD3, CD4, CD8, CD57 and granzyme B). Similar to previous work in this thesis they showed that CRC patients have a significant, T-stage dependent increase of FC. This reduced significantly post resection. [246]

However it remains unclear whether the above assessments of local inflammation relate to colonic inflammation measured by FC. FC may act as a surrogate marker of the overall inflammatory tumour microenvironment or as a marker of specific components.

If FC correlates with the tumour microenvironment it could allow a simple assessment of the LIR at an earlier point in cancer staging. An earlier understanding of the LIR, and what patients have high risk factors, could be used along with traditional staging to help dictate management. The use of neoadjuvant and adjuvant treatment could be adjusted based upon this additional staging information. [125]

I hypothesise that, in a cohort of patients with CRC, elevation in FC is associated with more inflammatory/ immunogenic tumour microenvironment. The aim of this pilot study was to compare FC with common markers of local inflammation or immune activity to determine if there is a potential association worthy of further study.

7.2 Methods

7.2.1 Patients

This chapter examined the same cohort of patients as chapter 6. All patients with CRC and FC results from both the 'Investigation of the local and systemic inflammatory response and of dietary habits in those attending for investigation via the National Health Service Colorectal Cancer Screening Programme', study initially examined in chapter 4, where it is described in more detail. As well as the additional FC patients, from earlier years (2011 to 2014), described in chapter 6.

These fifty six patients were cross referenced with the Academic Unit of Surgery's, University of Glasgow database of CRC patients with tumour microenvironment information. This resulted in eighteen patients with CRC, with FC and TME information. These eighteen patients subsequently had T-cell LIR assessed.

7.2.2 Methods

Faecal calprotectin was assessed per the same methods described in previous chapters 4-6. In this chapter, due to the unknown nature of FC in association with the LIR, I split FC into two sets of groups <50 µg/g and ≥50µg/g, <200µg/g and ≥200µg/g as well as using it as a continuous variable.

Assessment of the tumour microenvironment and TSP

The peritumoural inflammatory scores assessed were KM grade and TSP. KM grade has been described fully in section 1.6.4.2. TSP has been described fully in section 1.6.2.7.

The, adaptive immune response was assessed by examining T-cell infiltrate measured in the TME. The T-cells measured were CD3, CD68, CD8, FOXP3 and a combination if these cells (FOXP3/CD3, CD3/CD8, and CD68/CD3). Using Visiopharm® (pathology image analysis software) cores of the tumours were analysed and the T-cells in the stroma area were automatically counted. The program works in three sections. Firstly to detect the tumour and stroma according to panCK and alpha-SMA staining, secondly nuclear detection using the DAPI channel, and thirdly detection of the staining of each cell type. The counts were then standardised with the total cells to create a cell percentage for each T-cell type. The detection of cells is therefore dependent on the staining. The program can be trained to detect multi cell staining, according to the individual staining

7.2.3 Objectives

The primary objective for this pilot study was to compare FC with markers of the LIR to determine if there is an association.

7.2.4 Statistical Analysis

FC and T-cell percentages were displayed as medians, and interquartile range. FC was grouped as explained above. T-cell percentages were grouped as tertiles for this pilot analysis, until true high/low thresholds have been established. The data for peritumoural inflammatory scores and T-cells were plotted against FC using both boxplots when the data were grouped and scatter graphs with line of best fit when data were continuous. No other statistical analysis was performed due to the small number of patients analysed in this pilot study. Statistical analysis was performed using SPSS version 28.0 for Windows (IBM Corporation, Armonk, NY).

7.3 Results

Eighteen patients had FC and peritumoural inflammation scores assessed. Seventeen of patients also had T-cell infiltrates assessed. Of these patients 28% (n=5) had stage 1 disease, 22% (n=4) stage 2, and 50% (n=9) stage 3. 33% (n=6) had T1/2 tumours, 56% (n=10) had T3 and 11% (n=2) had T4.

7.3.1 Faecal calprotectin and the tumour microenvironment: peritumoural inflammation scores

Faecal calprotectin levels ranged from <30µg/g to 948µg/g. Median FC for the cohort was 305.5µg/g (125.2-476.8µg/g). Two patients (11%) had a FC <50µg/g, four patients (22%) had a FC between 50 and 200µg/g, and twelve patients (67%) had a FC ≥200µg/g.

7.3.1.1 Klintrup-Mäkinen Grade

Table 7.1a describes the relationship between KM and FC at a cut-off of 50µg/g. Patients with a high KM grade (2/3) have a higher median FC (389µg/g) in comparison to those with a low KM grade (0/1) with a median FC of 275µg/g. When the KM grade was considered as the four point score, KM 0 had the lowest median FC value (252µg/g), increasing to 668µg/g in KM 3. Two patients, have a FC <50µg/g. Both have a low KM grade. Table 7.1b describes the relationship between KM and FC at a cut-off of 200µg/g. Six patients have a FC <200µg/g and again all are in the low KM group.

Figures 7.1a and b display KM grade and FC in a boxplot graph. This displays the generalised higher FC levels in high grade compared to low grade KM.

7.3.1.2 Tumour Stroma Percentage

Table 7.1a describes the relationship between TSP and FC at a cut-off of 50µg/g. Low TSP patients have a median FC of 264µg/g, whereas high TSP patients have a median FC of 480µg/g. The two patients with a FC <50µg/g are both in the low TSP group. Table 7.1b describes the relationship between TSP and FC at a cut-off of 200µg/g. The six patients with a FC <200µg/g are all in the low TSP group.

Figure 7.2 displays TSP and FC in a boxplot graph. This displays the generalised higher FC levels in high TSP compared to low TSP.

Despite the small cohort size, these results suggest there may be an association between FC, KM and TSP.

7.3.2 Faecal calprotectin and the tumour microenvironment: T-cell infiltrate

7.3.2.1 CD3

Table 7.2a displays the relationship between CD3 and FC at a cut-off of 50µg/g. The median FC for this cohort is 336µg/g. The median FC is highest in the highest tertile at 479µg/g. The two patients with a FC <50 µg/g are in the two lowest tertiles. The median cell percentage for CD3 is 11.2, this is lower at 7.6 in the patients with a FC<50µg/g. Table 7.2b describes the relationship between CD3 and FC at a cut-off of 200µg/g, with no difference in median cell percentage between the two groups. Figure 7.3 displays CD3 and FC in a boxplot graph. This shows the highest tertile generally has the highest FC, but the middle tertile has lower FC compared to the lowest tertile. Figure 7.4 displays CD3 and FC in a scatter graph. Overall this shows a tendency for higher CD3 cell percentages to be associated with higher FC levels.

7.3.2.2 CD68

Table 7.2a displays the relationship between CD68 and FC at a cut-off of 50µg/g. Median FC is highest in the lowest tertile at 446µg/g. The two patients with a FC <50 µg/g are in the two highest tertiles. The median cell percentage for CD68 is 3.9, this is higher at 6.6 in the patients with a FC<50µg/g, and lower at 3.7 if FC ≥50 µg/g. Table 7.2b describes the relationship between CD68 and FC at a cut-off of 200µg/g. There is no difference in median cell percentage between patients with a FC less or more than 200µg/g. Figures 7.5 displays CD68 and FC in a boxplot graph. Figure 7.6 displays CD68 and FC in a scatter graph. Overall there is a wide range of distribution but tendency for lower CD68 cell percentages to be associated with higher FC levels.

7.3.2.3 CD8

Table 7.2a displays the relationship between CD8 and FC at a cut-off of 50µg/g, and table 7.2b a FC cut-off of 200µg/g. Only thirteen patients had CD8 results, and of these patients and of these eight were scored 0. Therefore no groupings were created. One patients had a FC <50 µg/g. Four patients have a FC<200µg/g. Figure 7.7 displays CD8 and FC in a scatter graph.

7.3.2.4 FOXP3

Only three patients had a FOXP3 score, one of which was 0. Therefore no analysis was performed on this group.

7.3.2.5 FOXP3/CD3

Table 7.2a displays the relationship between FOXP3/CD3 and FC at a cut-off of 50µg/g. Median FC is highest in the lowest tertile at 507.5µg/g. The median cell percentage for FOXP3/CD3 is similar in the patients with a FC more and less than 50µg/g. Table 7.2b describes the relationship between FOXP3/CD3 and FC at a cut-off of 200µg/g. Patients with a FC<200µg/g had a higher median cell percentage at 4.6, in comparison to 3.0 in those with FC≥200µg/g.

Figures 7.8 displays FOXP3/CD3 and FC in a boxplot graph. This shows higher FC levels in the lowest tertile, and lower levels in the middle and highest tertile. Figure 7.9 displays FOXP3/CD3 and FC in a scatter graph, which shows the majority of patients with a FOXP3/CD3<10, with a wide range of FC values.

7.3.2.6 CD3/CD8

Table 7.2a displays the relationship between CD3/CD8 and FC at a cut-off of 50µg/g. Seven patients have a score of 0. Median FC is highest in the highest tertile at 472.5µg/g. Table 7.2b describes the relationship between CD3/CD8 and FC at a cut-off of 200µg/g. Patients with a FC<200µg/g had a higher median cell percentage at 0.1, in comparison to 0.05 in those with FC≥200µg/g.

Figure 7.10 displays CD3/CD8 and FC in a boxplot graph. This shows similar distribution of the IQRs between the tertiles, but with a larger range in the highest tertile. Figure 7.11 displays CD3/CD8 and FC in a scatter graph. This shows the majority of CD3/CD8 cell percentage <1.0, but with two outliers ≥1.5.

7.3.2.7 CD68/CD3

Table 7.2a displays the relationship between CD68/CD3 and FC at a cut-off of 50µg/g. Median FC is highest in the highest tertile at 389µg/g. The median cell percentage for CD68/CD3 is 1.0, this is higher in the patients with a FC less than 50µg/g (1.3 vs 1.0). Table 7.2b describes the relationship between CD68/CD3 and FC at a cut-off of 200µg/g. Patients with a FC<200µg/g had a lower median cell percentage at 0.1, in comparison to 0.9 in those with FC≥200µg/g.

Figures 7.12 displays CD68/CD3 and FC in a boxplot graph. This shows both the highest FC levels, and the largest range in the highest tertile. Figure 7.13 displays CD68/CD3 and FC in a scatter graph. This shows a tendency for higher CD68/CD3 cell percentages to be associated with higher FC levels.

Overall these numbers are obviously very small to allow any analysis, and overall shows a lot of heterogeneity between FC and T-cell values.

7.4 Discussion

In this chapter I examined the relationship between FC and tumour microenvironment. To summarise, in this pilot study the overall numbers are too small to allow any significance to be determined. However they do suggest that there may be an association between FC, KM and TSP.

I found that both KM and TSP have higher FC levels in high grade KM and TSP, in comparison to low grade. KM is a measure of the generalised density of inflammatory cells. Therefore an association between KM and FC could imply either that FC is a true reflection of the generalised LIR in the TME, or that it is a marker of an individual component, but this is reflected in KM. [296]

Calprotectin is a complex of S100 proteins, S100A8 and S100A9, which are low-molecular weight members of the S100 family expressed in cells of the myeloid lineage, including monocytes, neutrophils and macrophages. These inflammatory proteins have been reported to promote tumourigenesis, as well as causing metastatic spread. Many studies (in multiple cancers including pancreatic, prostate, oral and breast as well as CRC) have reported the stroma of tumours contains high numbers of the S100A8 and S100A9 proteins, associated with reduced cancer specific survival. [296-300] Therefore it may be that based upon S100A8/9 correlation with tumour stroma and survival, that FC will also have an association with TSP in CRC.

S100A8 has been shown to be highly expressed in TILs with range in numbers from low to high density. S100A9 has also been detected in the TIL cells of tumour stroma. [301] Of the T-lymphocytes assessed in this study the small numbers made analysis difficult, and overall showed a lot of heterogeneity between FC and T-cell values. However CD3 showed the closest association with higher FC levels.

In this study there was a tendency for lower CD68 cell percentages to be associated with higher FC levels. It has also been shown that calprotectin reactivity was found mainly in granulocytes and macrophage. [251, 292] This has been thought secondary to shedding from ulcerated tumour. [246] CD68 is a common macrophage marker. Therefore these results are the converse of what you may hypothesise.

Calprotectin detected in faeces is a measure of colonic inflammation, it is found predominantly in the cytoplasm of neutrophils and the membrane of monocytes and is released upon neutrophil cell death or damage. [206-208, 210] In IBD, at the acute stage of mucosal inflammation, there is formation of cryptitis and abscess. This results from the subsequent influx of neutrophils into the bowel lumen. FC levels have also been shown to be proportional to mucosal neutrophilic infiltration. Similarly, in CRC, it has been found that neutrophils infiltrate neoplastic tissue as per the volume of the neoplasm, with Luley et al showing a significant correlation between mucosal

calprotectin and neutrophil infiltration. [213, 294, 302, 303, 292] Are increased FC levels in CRC therefore due to neutrophil infiltration and calprotectin production, secondary to mucosal disruption? [292] Would FC levels correlate with tumour associated neutrophils (TANs)? TANs are thought to have both tumour supportive and suppressive functions, and have been found to increase overall survival but can promote metastatic spread. [304, 305]

As previously mentioned, the major limitation of this study is the size of study cohort. As a pilot study, it has shown both that work of this type can be performed and that there may be associations between FC and the LIR. The second limitation is the novel strategy used to assess the different T-cell lymphocytes. This is an alternative assessment of T-cells, compared to the traditional chromogenic immunohistochemistry as used in the Immunoscore. However this automated assessment provides reliable quantification, and may be a faster, less error prone assessment of T-cells.

It would be of interest to expand this pilot study to assess the relationship between FC and markers of the LIR. It would also be of interest to correlate FC with patient outcome data.

In conclusion this pilot study has shown that there may be an association between FC and local peritumoural inflammation in the tumour microenvironment in CRC. This warrants further study within a larger cohort of patients.

7.5 Tables and Figures

Table 7.1a Faecal Calprotectin and peritumoural inflammation (FC cut-off 50µg/g**)**

	Score	n (%)	Median FC µg/g (IQR)	FC<50 n (%)	FC ≥50 n (%)
Total		18	305.5 (125.3-476.8)	2 (11)	16 (89)
KM Grade	Low	15 (83)	275 (120-446)	2 (13)	13 (87)
	High	3 (17)	389	0 (0)	3 (100)
KM Score	0	4 (22)	252 (152.5-403.3)	0 (0)	4 (100)
	1	11 (61)	336 (98-569)	2 (18)	9 (82)
	2	1 (6)	-	0 (0)	1 (100)
	3	2 (11)	668	0 (0)	2 (100)
TSP	Low	16 (89)	264 (121.8-437)	2 (13)	14 (88)
	High	2 (11)	480	0 (0)	2 (100)
T-stage	1/2	6 (33)	266 (97.3-571.5)	1 (17)	5 (83)
	3	10 (56)	306 (196.3-449.8)	1 (10)	9 (90)
	4	2 (11)	376	0 (0)	2 (100)
N-stage	0	9 (50)	336 (138-513)	1 (11)	8 (89)
	1	9 (50)	275 (112.5-489.5)	1 (11)	8 (89)
Stage	1	5 (28)	156 (74.5-411)	1 (20)	4 (80)
	2	4 (22)	458 (255.8-613)	0 (0)	4 (100)
	3	9 (50)	275 (112.5-489.5)	1 (11)	8 (89)

Table 7.1a Faecal calprotectin and peritumoural inflammation (FC cut-off 50 µg/g)

Table 7.1b Faecal Calprotectin and peritumoural inflammation (FC cut-off 200µg/g**)**

	Score	n (%)	Median FC µg/g (IQR)	FC<200 n (%)	FC ≥200 n (%)
Total		18	305.5 (125.3-476.8)	6 (33)	12 (67)
KM Grade	Low	15 (83)	275 (120-446)	6 (40)	9 (60)
	High	3 (17)	389	0 (0)	3 (100)
KM Score	0	4 (22)	252 (152.5-403.3)	1 (25)	3 (75)
	1	11 (61)	336 (98-569)	5 (46)	6 (55)
	2	1 (6)	-	0 (0)	1 (100)
	3	2 (11)	668	0 (0)	2 (100)
TSP	Low	16 (89)	264 (121.8-437)	6 (38)	10 (63)
	High	2 (11)	480	0 (0)	2 (100)
T-stage	1/2	6 (33)	266 (97.3-571.5)	3 (50)	3 (50)
	3	10 (56)	306 (196.3-449.8)	2 (20)	8 (80)
	4	2 (11)	376	1 (50)	1 (50)
N-stage	0	9 (50)	336 (138-513)	3 (33)	6 (67)
	1	9 (50)	275 (112.5-489.5)	3 (33)	6 (67)
Stage	1	5 (28)	156 (74.5-411)	3 (60)	2 (40)
	2	4 (22)	458 (255.8-613)	0 (0)	4 (100)
	3	9 (50)	275 (112.5-489.5)	3 (33)	6 (67)

Table 7.1b. Faecal calprotectin and peritumoural inflammation (FC cut-off 200 µg/g)

Table 7.2a Faecal Calprotectin and peritumoural T-cells (FC cut-off 50µg/g**)**							
	Total n (%)	Median cell % (IQR)	Median cell % (IQR) *if FC<50*	Median cell % (IQR) *if FC ≥50*	FC<50 n (%)	FC ≥50 n (%)	Median FC (µg/g)
CD3	17[a]	11.2 (4.9-14.4)	7.6	11.2 (6.3-15.9)	2 (12)	15 (88)	336 (123.5-507.5)
0.9-8.9	6 (35)	-	-	-	1 (17)	5 (83)	294 (180-419)
9.0-12.69	5 (29)	-	-	-	1 (20)	4 (80)	127 (63.5-449.5)
12.7-37.9	6 (35)	-	-	-	0 (0)	6 (100)	479 (312-672)
CD68	17[a]	3.9 (2.6-6.7)	6.6	3.7 (2.4-6.5)	2 (12)	15	336 (123.5-507.5)
0.8-3.68	5 (29)	-	-	-	0 (0)	5 (100)	446 (248-764)
3.7-5.5	7 (41)	-	-	-	1 (14)	6 (86)	275 (98-389)
5.6-8.6	5 (29)	-	-	-	1 (17)	5 (83)	252 (129-596.5)
CD8	13[a]	0.0 (0.0-0.1)	0.0	0.0 (0.0-0.1)	1 (8)	12 (92)	376 (123.5-574.5)
FOXP3/CD3	17[a]	3.5 (2.2-6.1)	3.8	3.2 (2.2-7.0)	2 (12)	15 (88)	336 (123.5-507.5)
0.5-2.8	6 (35)	-	-	-	0 (0)	6 (100)	507.5 (354.8-705)
2.9-4.558	5 (29)	-	-	-	2 (40)	3 (60)	98.0 (31.5-356)
4.6-51.1	6 (35)	-	-	-	0 (o)	6 (100)	252 (125.3-452.5)
CD3/CD8	17[a]	0.1 (0.0-0.4)	0.08	0.1 (0.0-0.4)	2 (12)	15 (88)	336 (123.5-507.5)
0.0-0.0	7 (41)	-	-	-	1 (14)	6 (86)	336 (229-410)
0.1-0.164	4 (24)	-	-	-	0 (0)	4 (100)	201 (105.3-536.8)
0.165-2.9	6 (35)	-	-	-	1 (17)	5 (83)	472.5 (97.3-672)
CD68/CD3	17[a]	1.0 (0.6-1.9)	1.3	1.0 (0.6-1.8)	2 (12)	15 (88)	336 (123.5-507.5)
0.0-0.655	5 (29)	-	-	-	1 (20)	4 (80)	252 (131.5-513)
0.656-1.4	7 (41)	-	-	-	0 (0)	7 (100)	175 (120-410)
1.5-3.1	5 (29)	-	-	-	1 (20)	4 (80)	389 (202.5-786)

Table 7.2a. Faecal Calprotectin and peritumoural T-cells (FC cut-off 50µg/g)
[a] Number of patients when incomplete data available

Table 7.2b Faecal Calprotectin and peritumoural T-cells (FC cut-off 200µg/g**)**							
	Total n (%)	Median cell % (IQR)	Median cell % (IQR) if FC<200	Median cell % (IQR) if FC ≥200	FC <200 n (%)	FC ≥200 n (%)	Median FC (µg/g)
CD3	17[a]	11.2 (4.9-14.4)	11.2 (7.2-12.3)	10.7 (3.9-16.6)	5 (29)	12 (71)	336 (123.5-507.5)
0.9-8.9	6 (35)	-	-	-	1	5	294 (180-419)
9.0-12.69	5 (29)	-	-	-	3	2	127 (63.5-449.5)
12.7-37.9	6 (35)	-	-	-	1	5	479 (312-672)
CD68	17[a]	3.9 (2.6-6.7)	3.9 (2.2-6.6)	4.2 (2.5-6.8)	5 (29)	12 (71)	336 (123.5-507.5)
0.8-3.68	5 (29)	-	-	-	1	4	446 (248-764)
3.7-5.5	7 (41)	-	-	-	3	4	275 (98-389)
5.6-8.6	5 (29)	-	-	-	1	4	252 (129-596.5)
CD8	13[a]	0.0 (0.0-0.1)	0.0 (0.0-0.0)	0.05 (0.0-0.4)	4 (31)	9 (69)	376 (123.5-574.5)
FOXP3/CD3	17[a]	3.5 (2.2-6.1)	4.6 (3.8-7.7)	3.0 (2.1-5.1)	5 (29)	12 (71)	336 (123.5-507.5)
0.5-2.8	6 (35)	-	-	-	0	6	507.5 (354.8-705)
2.9-4.558	5 (29)	-	-	-	3	2	98.0 (31.5-356)
4.6-51.1	6 (35)	-	-	-	2	4	252 (125.3-452.5)
CD3/CD8	17[a]	0.1 (0.0-0.4)	0.1 (0.05-0.3)	0.05 (0.0-0.6)	5 (29)	12 (71)	336 (123.5-507.5)
0.0-0.0	7 (41)	-	-	-	1	6	336 (229-410)
0.1-0.164	4 (24)	-	-	-	2	2	201 (105.3-536.8)
0.165-2.9	6 (35)	-	-	-	2	4	472.5 (97.3-672)
CD68/CD3	17[a]	1.0 (0.6-1.9)	0.1 (0.7-1.6)	0.9 (0.5-2.0)	5 (29)	12 (71)	336 (123.5-507.5)
0.0-0.655	5 (29)	-	-	-	1	4	252 (131.5-513)
0.656-1.4	7 (41)	-	-	-	3	4	175 (120-410)
1.5-3.1	5 (29)	-	-	-	1	4	389 (202.5-786)

Table 7.2b. Faecal Calprotectin and peritumoural T-cells (FC cut-off 200µg/g)
[a] Number of patients when incomplete data available

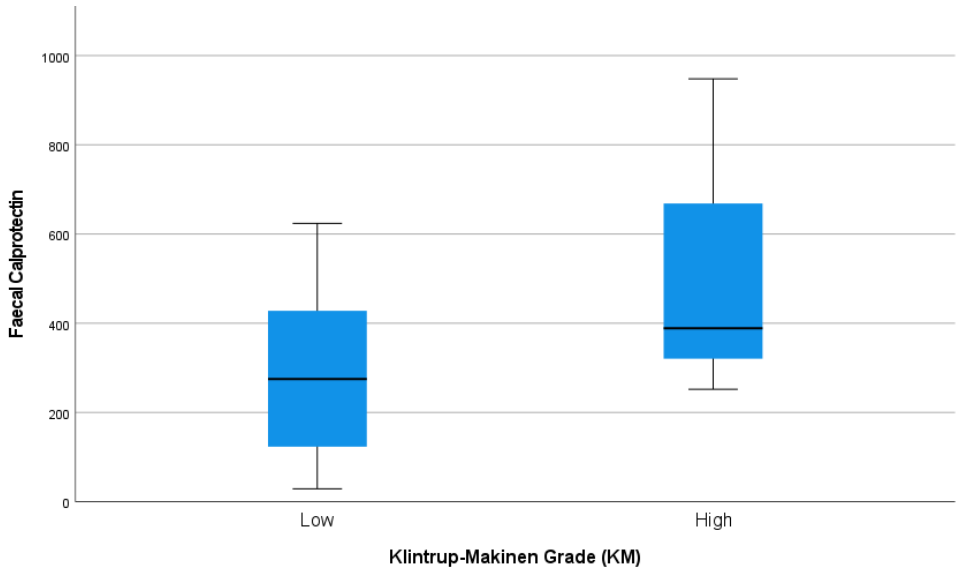

Figure 7.1a. Klintrup-Makinen Grade and Faecal Calprotectin Boxplot

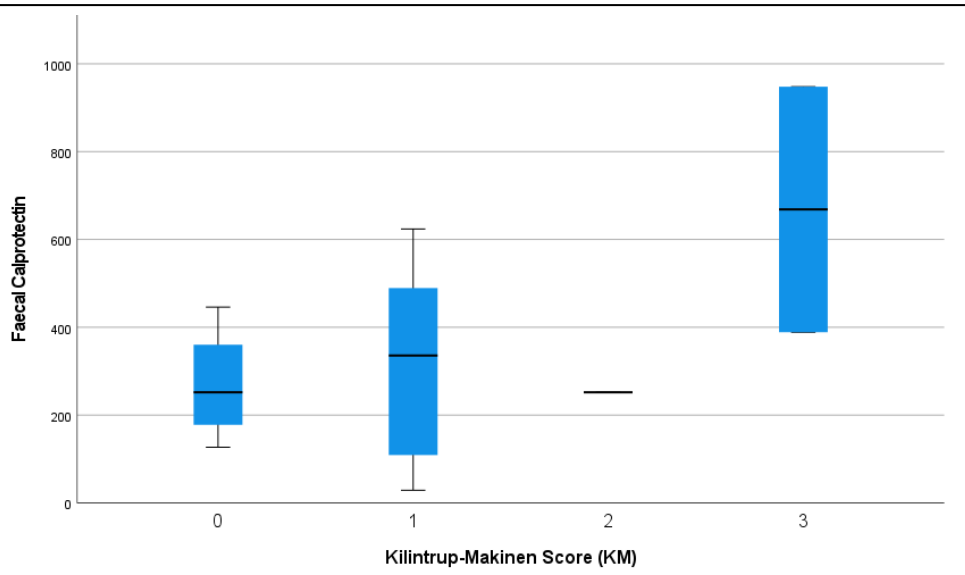

Figure 7.1b. Klintrup-Makinen Score and Faecal Calprotectin Boxplot

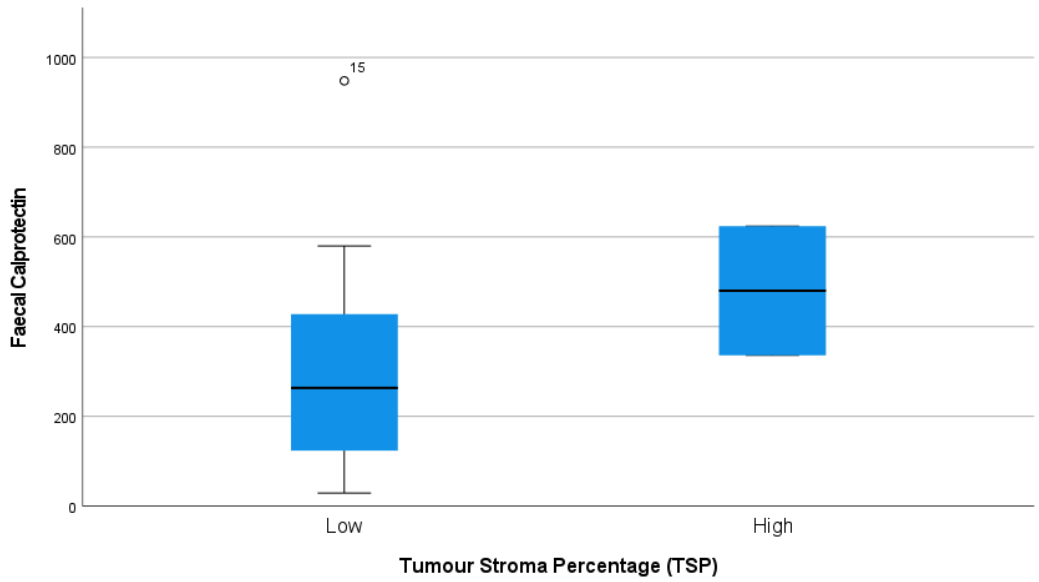

Figure 7.2 Tumour Stroma Percentage and Faecal Calprotectin Boxplot

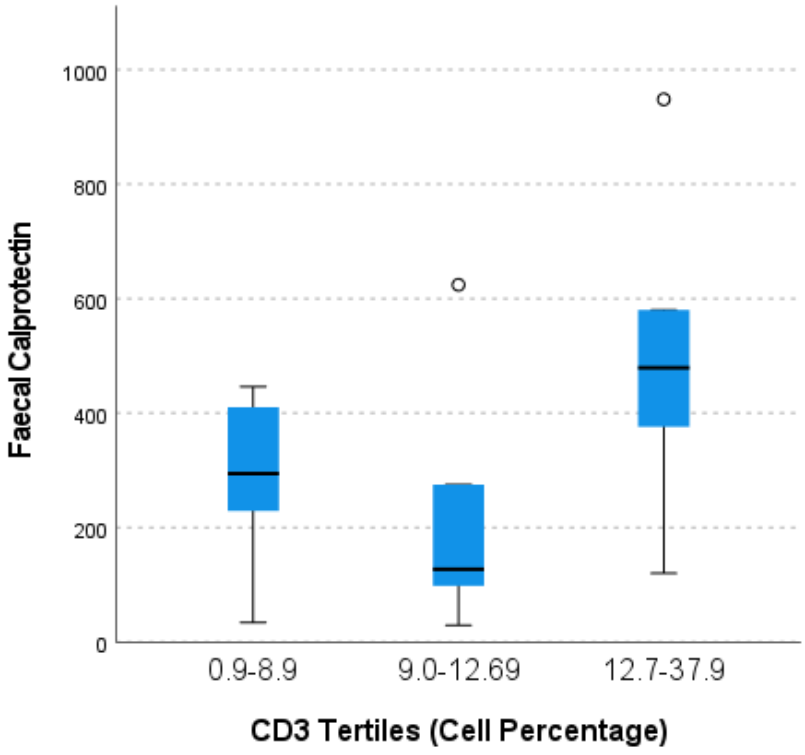

Figure 7.3. CD3 and Faecal Calprotectin Boxplot

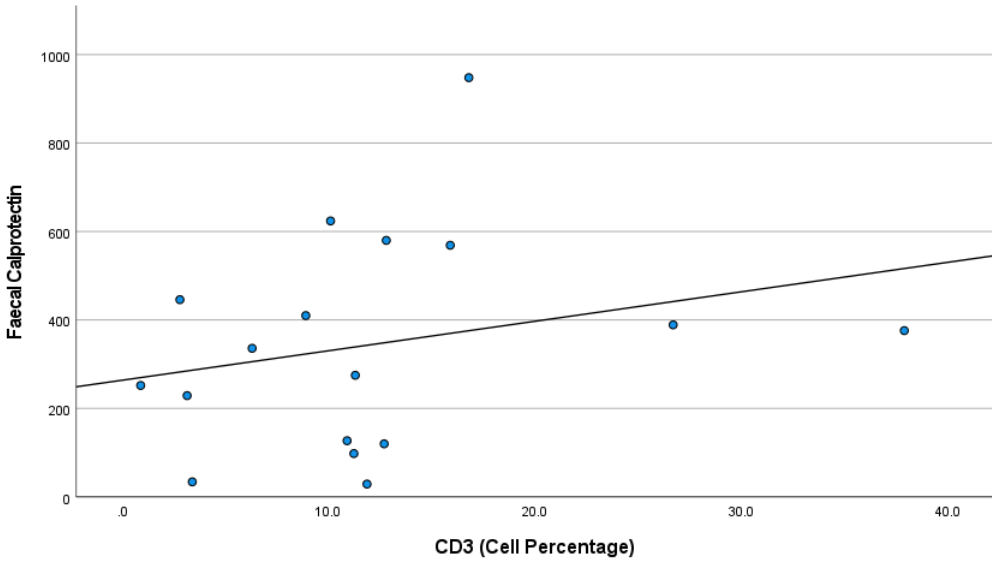

Figure 7.4. CD3 and Faecal Calprotectin Scatter Graph

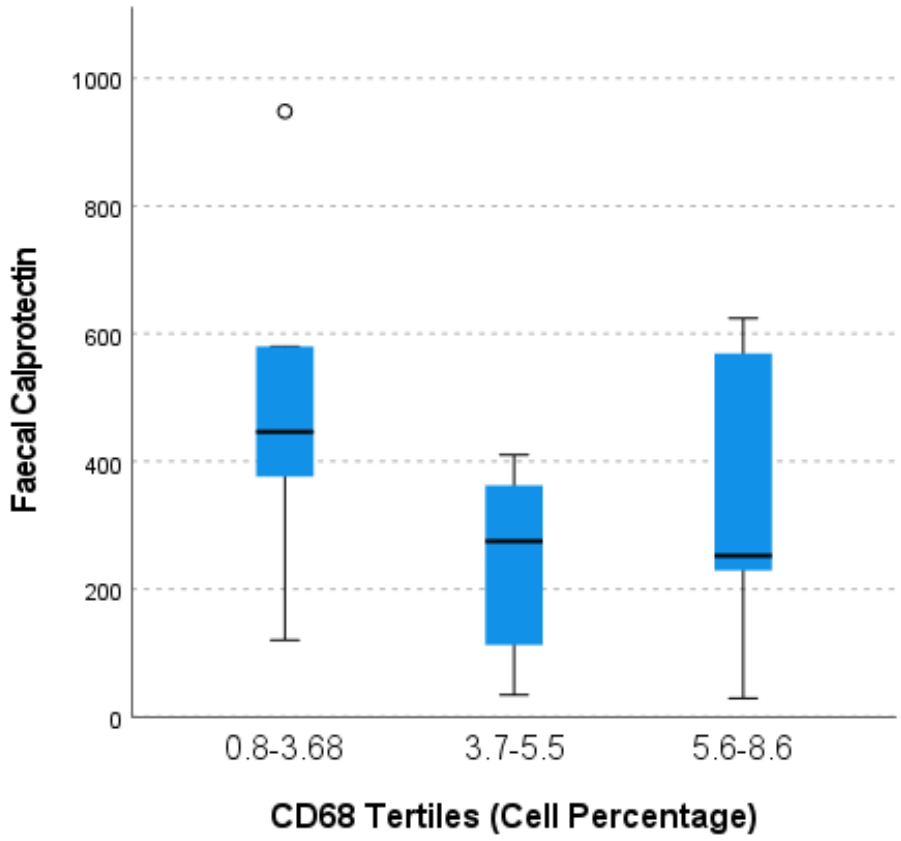

Figure 7.5. CD68 and Faecal Calprotectin Boxplot

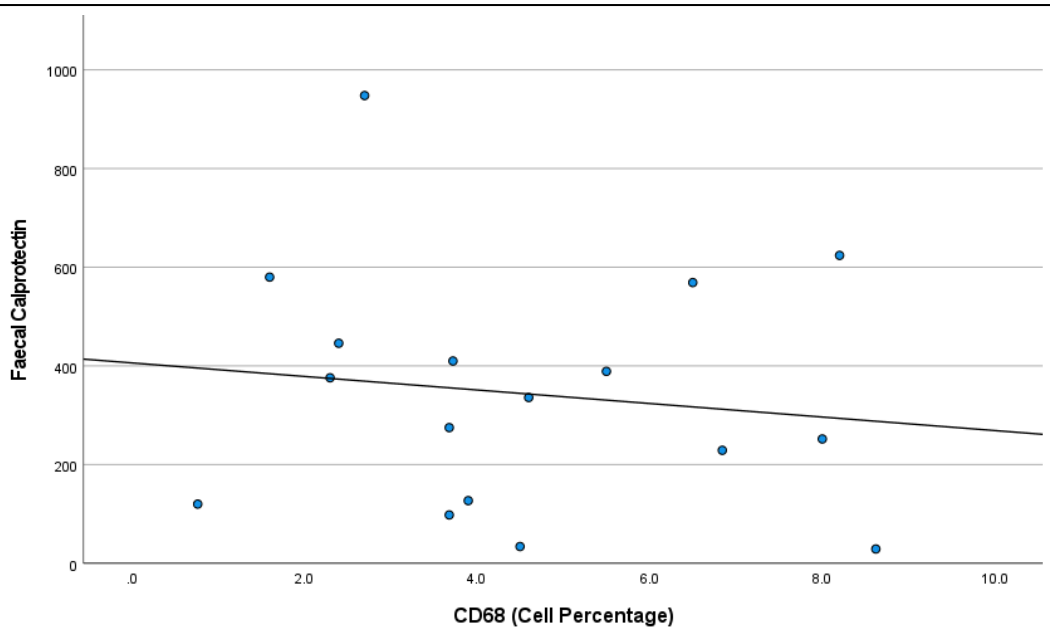

Figure 7.6. CD68 and Faecal Calprotectin Scatter Graph

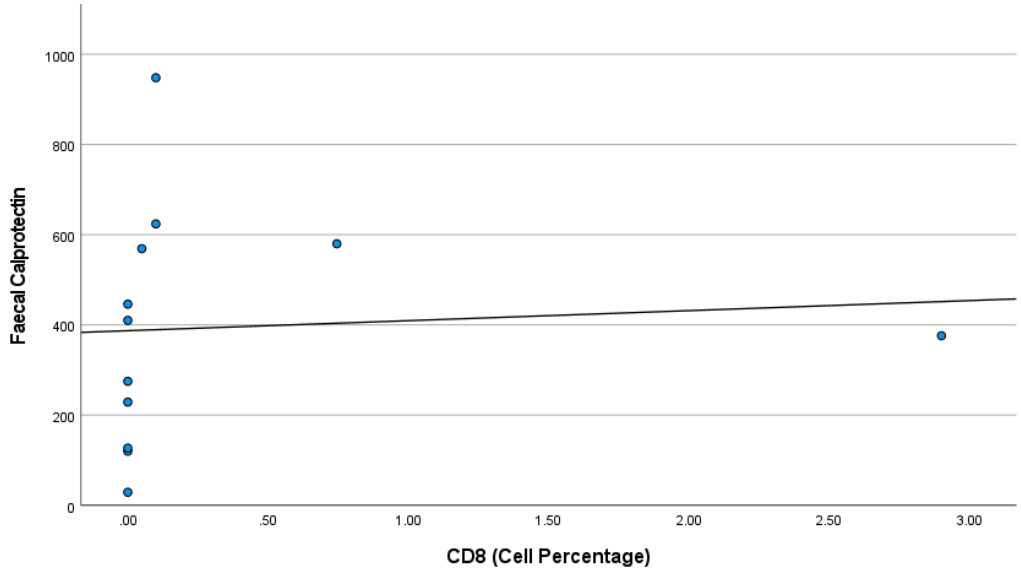

Figure 7.7. CD8 and Faecal Calprotectin Scatter Graph

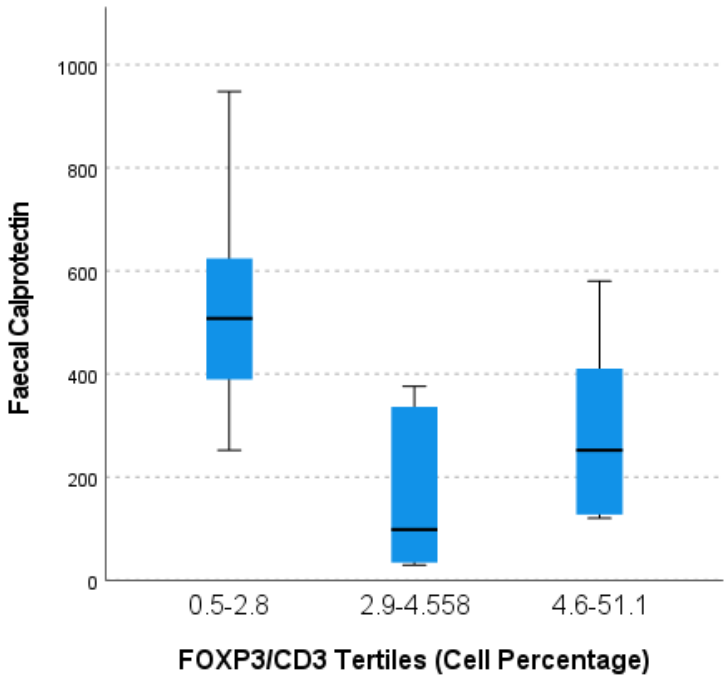

Figure 7.8. FOXP3/CD3 and Faecal Calprotectin Boxplot

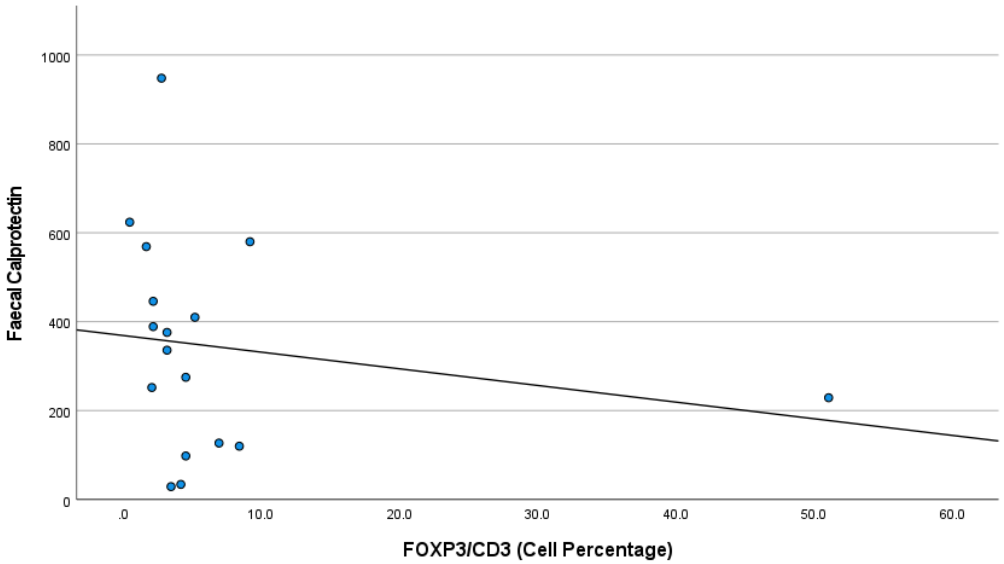

Figure 7.9 FOXP3/CD3 and Faecal Calprotectin Scatter Graph

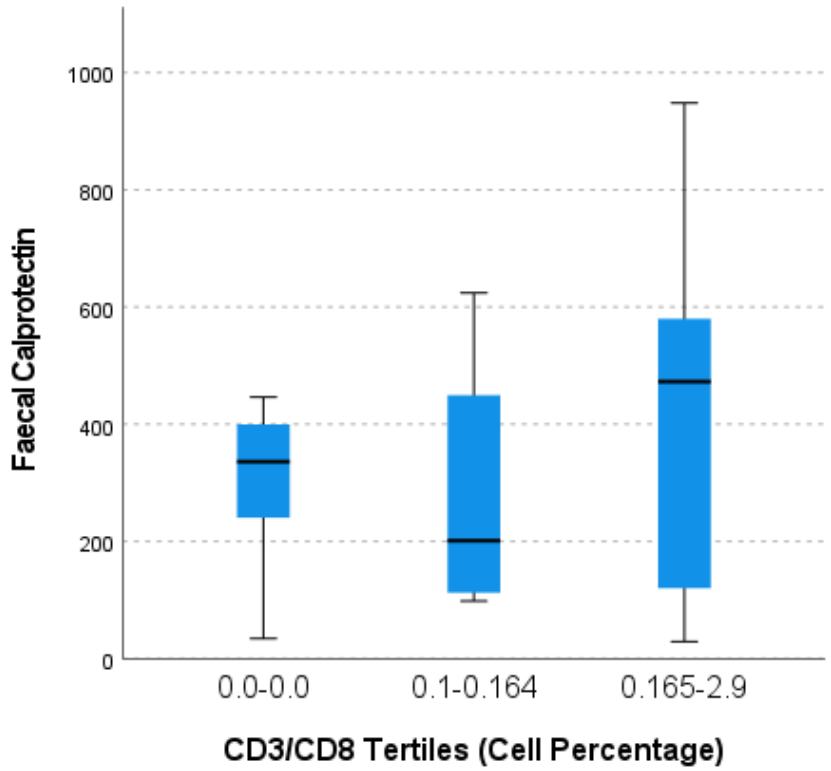

Figure 7.10. CD3/CD8 and Faecal Calprotectin Boxplots

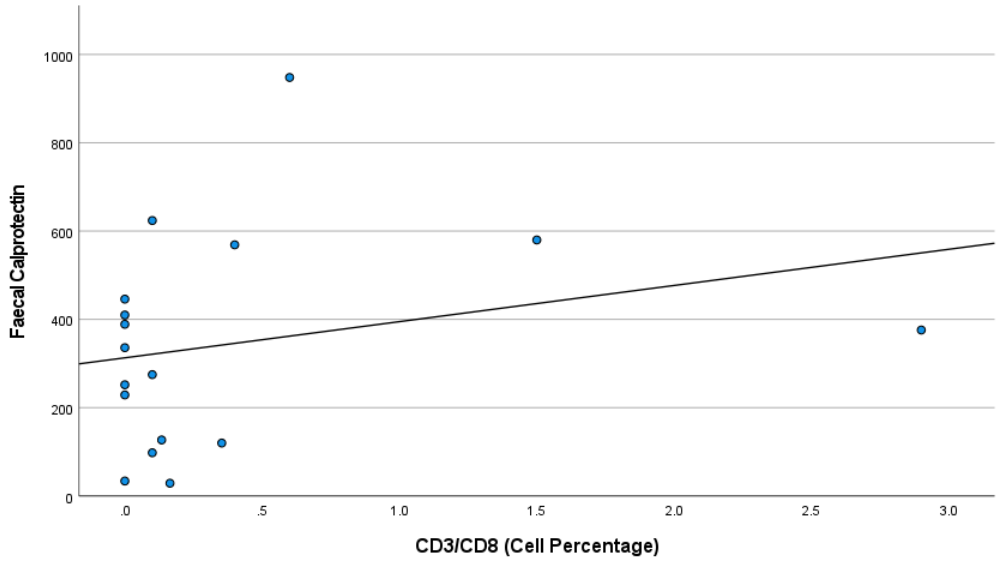

Figure 7.11. CD3/CD8 and Faecal Calprotectin Scatter Graph

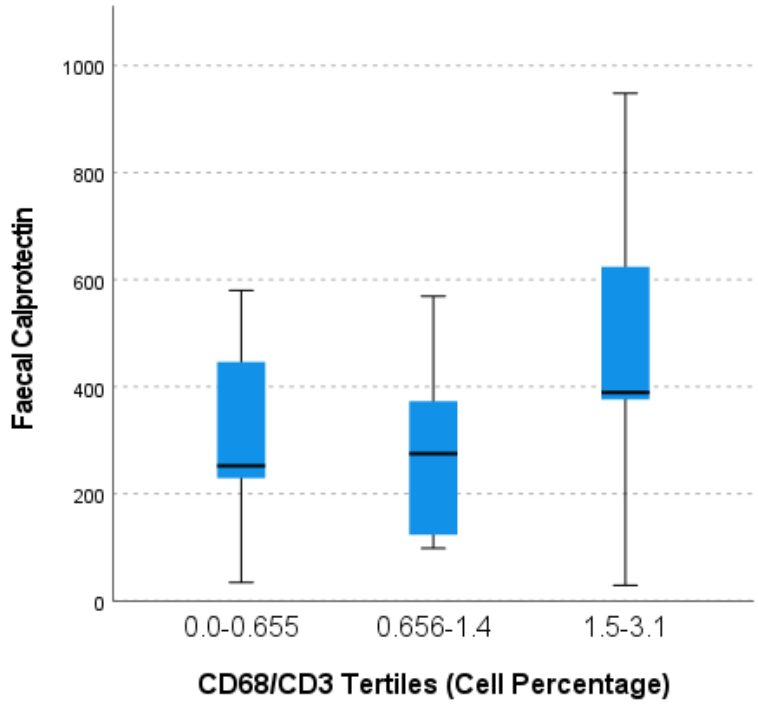

Figure 7.12. CD68/CD3 and Faecal Calprotectin Boxplots

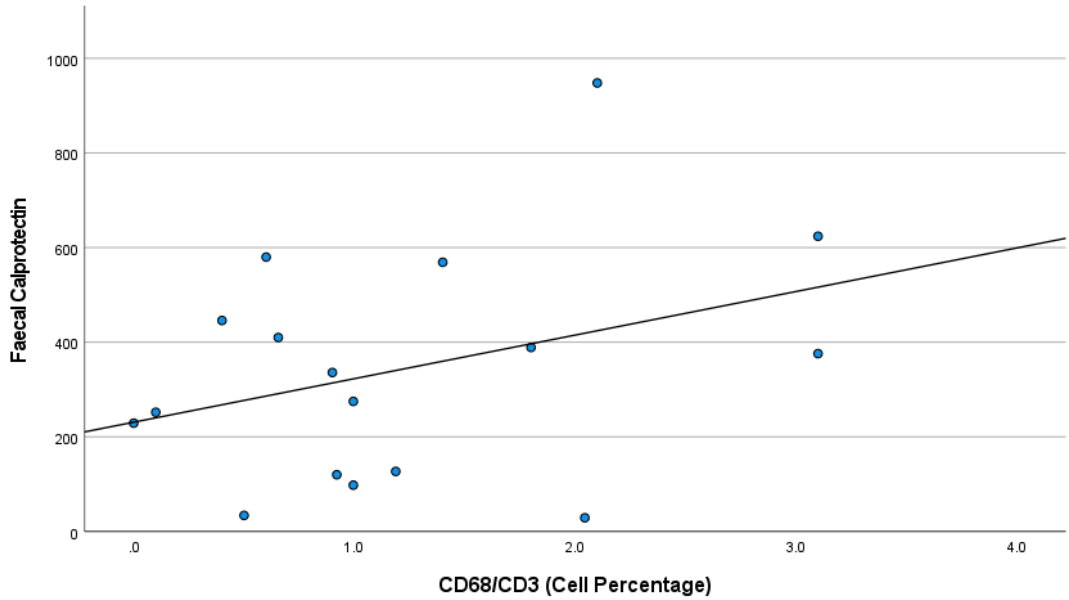

Figure 7.13. CD68/CD3 and Faecal Calprotectin Scatter Graph

8.0 Conclusions

8.1 Overview of work

Initially, it was recognised that while the staging and prognosis of CRC is currently still based upon the classical tumour, nodes, metastases assessment, that the role of both local colonic inflammation and systemic inflammation are also important components of determining cancer progression and survival.

The presence of an inflammatory tumour microenvironment is recognised as the seventh hallmark of cancer. The relationships between the presence of a systemic inflammatory response and colorectal cancer development is well described. Within the tumour microenvironment, conflicting pro-tumour and anti-tumour inflammatory responses can dictate cancer outcomes. The presence of a higher stromal to epithelial volume within the tumour is associated with presence of immune-suppressive pro-cancer inflammation and poorer cancer outcomes. Conversely a pronounced local inflammatory response characterised by a high grade lymphocytic infiltrate within the tumour microenvironment is associated with improved cancer outcomes. The local inflammatory response in the tumour microenvironment is one such area which may be targetable for modification, possibly at a neoadjuvant stage. Present methods of assessing the tumour microenvironment rely on tissue sampling, and are typically reliant on post-operative resection specimens. Despite the acknowledged prognostic relationship, assessment of the local inflammatory response is currently not a requirement of in staging of CRC.

Calprotectin detected in the faeces is a sensitive measure of intraluminal colonic inflammation and is currently well established in the clinical assessment of IBD, this therefore represents another assessment of inflammation. At the outset of this research it is unknown whether colonic inflammation measured by FC, is a marker of CRC and whether it has a role in the development or progression of CRC is not known.

In chapter 3, a systematic review and meta-analysis, confirmed that the role of FC in the diagnosis of CRC has not been defined. There is a lack of evidence supporting an association between FC and adenoma/advanced adenomas. However my review confirmed an associated between FC and CRC with median FC found to be higher in CRC, in comparison to healthy subjects in fifteen out of the sixteen studies and a 5-fold increased likelihood than controls to have an elevated FC (OR 5.19, 95% CI 3.12-8.62, P<0.001 with a heterogeneity (I^2=27%)). There was minimal literature on how FC correlates with location, stage, histology or inflammation in CRC.

In chapter 4, I studied the role of FC in a large well defined cohort of FOBT positive patients as part of a screened population. In this study, on multivariate analysis, aspirin use (OR 2.21, 95% CI 1.15-4.24, p<0.05), NSAID use (OR 2.41, 95% CI 1.14-5.12, p<0.05), PPI use were independently

associated with FC >50µg/g. A higher FC was associated with inflammatory ($p<0.05$) pathology which is well recognised and also with CRC ($p<0.05$). FC was strongly associated with CRC (sensitivity 92.8% for CRC, at 50µg/g) but lacked specificity. FC also failed to show sufficient sensitivity and specificity for the detection of non-cancer neoplasia.

In this screening cohort, in chapter 5, I evaluated the relationships between FC and systemic markers of inflammation. Patients with an inflammatory pathology were shown to be more likely to be systemically inflamed. There was no evidence of a strong link between a SIR and presence of colorectal neoplasia. There was no significant relationship seen between FC and SIR.

In chapter 6, I studied a larger cohort of colorectal cancer patients in whom all had FC measurements. This showed that advanced disease stage was non-significantly associated with higher levels of FC, with T4 tumours having the highest median FC (321µg/g), with 67% having a FC ≥200µg/g. 29% of those with T1 tumours had a FC ≥200µg/g. Patients with nodal or metastatic disease had non-significantly higher median FC, compared to those without. Patients with peritoneal involvement had significantly higher median FC, compared to those without, median FC (405µg/g vs 164µg/g), $p<0.05$. 89% of patients with peritoneal involvement had FC ≥200µg/g, compared to 44% in those without peritoneal involvement ($p<0.05$). Poorly differentiated tumours had a higher median FC (281.5µg/g) than well/moderate differentiated tumours (169µg/g), but not significantly. Patients with larger tumour had non-significantly higher FC levels, tumours ≥3.5cm had a higher median FC 251.5µg/g, and 67% had a FC ≥200µg/g, in comparison to those with a tumour <3.5cm (median 164 µg/g, and 48% FC ≥200µg/g). To summarise there may be a potential relationship between higher levels of FC and colorectal cancers with larger, more advanced tumours, displaying negative prognostic factors but more work is required to clarify this.

In a pilot study, in chapter 7, I assessed FC and markers of the LIR, looking for an association between FC and local peritumoural inflammation in the tumour microenvironment in CRC. The markers and cells assessed were KM grade, TSP, CD3+, CD8+ CD68+ and FOXP3+. I found that both KM and TSP have higher FC levels in high grade KM and TSP, in comparison to low grade. There was no clear relationship between FC and T-cells.

In summary this thesis has confirmed an association between FC and CRC, specifically in larger, more advanced tumours in CRC. There may be an association between FC and local peritumoural inflammation in the tumour microenvironment in CRC.

8.2 Future work

This thesis adds to the literature on FC in colorectal neoplasia, confirming a relationship with CRC.

Future study is of interest. Firstly, it would be of interest to expand the pilot study in chapter 7 to assess and validate the association between FC and local peritumoural inflammation in the tumour microenvironment in CRC. Ideally a large cohort study of patients with CRC would be recruited, perhaps through a similar mechanism to the work with the bowel screening cohort in this thesis. This would allow FC levels to be recorded early in the CRC pathway. These patients would then have the TME formally assessed including established peritumoural inflammatory scores such as KM Grade, The Immunoscore and GMS (and TSP as an individual entity) as well as T-cells, to allow a comprehensive analysis to be performed. If FC does correlate with the tumour microenvironment it could considered a non-invasive biomarker for enhanced immunogenicity within tumours. This could be of value in allowing a simple assessment of the LIR at an earlier point in cancer staging which would help to dictate neoadjuvant and adjuvant treatment. It may also be of value in the assessment/ trialling of response to immunomodulatory drugs in the future.

Secondly, the study above would allow further analysis of FC association with histological staging in CRC. I have shown that advanced CRC stage was associated with higher levels of FC. More patient numbers would allow a deeper analysis of FC correlation with CRC stage and whether it is not only is associated with advancing T-stage but further elevated in metastatic disease.

Finally, regarding its use in the prioritisation of patients requiring CRC diagnostic investigations and in the staging of CRC could be considered. I have shown that FC is highly sensitive for CRC, but at a specificity that renders in unsuitable to be used for population based screening. However in this Covid-19 era it is becoming increasingly beneficial to have methods to determine how urgently patients need to be investigated with symptoms suspicious of cancer, to ensure the limited resources available to the NHS are used to their fullest capacity. Larger cohort analysis of symptomatic patients would help to assess whether FC could be used safely in this way.

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Appendices

Appendix a.

Study Proposal. Version 4, 25/7/2016

Investigation of the local and systemic inflammatory response and of dietary habits in those attending for investigation via the National Health Service Colorectal Cancer Screening Programme

Research Student: Miss Cariss Little, Mr Domenic Di Rollo, Miss Fiona Ross

Supervisors: Mr. Campbell Roxburgh & Mr. David Mansouri - Departments of Surgery and Pathology, *Glasgow Royal Infirmary* (GRI) *and* Dr. Emilie Combet- Department of Nutrition, University of Glasgow.

A. Introduction

Colorectal cancer (CRC) is the 2nd most common cause of cancer-related death in the UK (2010) accounting for around 10% of all cancer deaths. In the UK, 41,000 new cases are diagnosed per year with 16,000 deaths.[306]·[307] Only a small percentage of cases of colorectal cancer arise in patients with known hereditary syndromes while the majority of cases are sporadic, arising due to multiple alterations and mutations promoting initiation and progression of the dysplasia-cancer sequence.[308, 91]

The Scottish Bowel Screening Programme was introduced across Scotland in a phased manner beginning in 2007. Men and women in Scotland between the ages of 50 and 74 years of age receive a Faecal Occult Blood test (FOBt) by post and if the overall result of the screening is positive, then the individual is referred to their local hospital and is offered a colonoscopy.[309] Its purpose is to reduce overall colorectal cancer mortality by increasing the number of early stage cancers diagnosed and treated with curative intent, therefore reducing cancer specific mortality.[310-312] For example, with current standard treatment reported 5 year survival rates for stages TNM I, II, II and IV disease are 93%, 77%, 48% and 7% respectively.[313] There is good evidence that screening for colorectal cancer increases the number of early-stage cancers diagnosed and the number of precancerous adenomas removed and as a result leads to a reduction of cancer specific mortality.[310, 314, 315] Despite the success of the colorectal cancer screening programme in downstaging colorectal cancer, interval cancer rates are substantial [316] and consequently there has been significant interest in chemoprevention to reduce a patients risk of colorectal cancer. Much of this work has concentrated on aspirin, ACE inhibitors and statins [317, 79] however, dietary compounds such as polyphenols have recently attracted attention as potential agents in the treatment of early stage colorectal cancer since they have low toxicity compared with current drugs. The Scottish diet is known to be typically low in fruit and vegetable and high in processed foods.[318, 319] Therefore polyphenols as well as other dietary components such as fibre[320] may be effective as part of a dietary recommendation for the general public alongside the colorectal cancer screening programme.

Inflammation has recently been recognised as the seventh hallmark of cancer and current evidence suggests a role for inflammation in the pathogenesis of CRC.[173] Inflammatory conditions in certain organs are known to increase the risk of cancer, however an inflammatory component has also been demonstrated in the microenvironment of tumours that do not arise on a background of a pre-existing inflammatory disorder. There is a body

of evidence to support the concept that infiltration of immune cells in the locality of the tumour is associated with improved clinical outcome in colorectal cancer, whilst the presence of a systemic inflammatory response (measured by circulating CRP levels) has been established as a predictor of recurrence and of overall survival.

Calprotectin is a neutrophil derived protein constituting approximately 60% of the soluble cytoplasmic proteins in neutrophilic granulocytes which can be quantified in the faeces and is accepted as a marked of gut inflammation.[321] It has been found in increased concentrations in those with colorectal cancer, inflammatory bowel disease and other inflammatory disorders.[322] Faecal calprotectin appears to be a more sensitive marker for CRC than FOBt but its specificity is probably too low for screening the general population.[323, 255] Furthermore, calprotectin levels have been found to be significantly higher in those with adenomas compared to those without.[255]

B. Aims

1. To investigate the impact of dietary components on the colonic health of people who present via the Scottish Bowel Screening Programme by administration of a food frequency questionnaire.

2. To investigate the effect of the inflammatory response in those attending for colonoscopy via the Scottish Bowel Screening Programme by measurement of faecal calprotectin and serum inflammatory markers (CRP, differential WCC, albumin, IL-6, serum calprotectin). We also wish to assess the local inflammatory response in any surplus tissue removed.

C. Research questions:

RQ1- Within a screened population is the diagnosis of colorectal neoplasia influenced by dietary components thought to affect bowel health e.g. polyphenols, fibre?

RQ2- Do patients who are diagnosed with a) colorectal adenomas or b) colorectal adenocarcinomas have higher levels of colonic inflammation (measured using faecal and serum calprotectin) than those without neoplasia? Does this vary with degree of dysplasia or stage of cancer at presentation?

RQ3- Do patients who are diagnosed with a) colorectal adenomas or b) colorectal adenocarcinomas following a positive FOBt as part of the Scottish Bowel Screening Programme have higher levels of systemic inflammation (measured using serum C-reactive protein, albumin and a differential white cell count) than those without neoplasia?

RQ4- Is there any relationship between fibre/ polyphenol intake and local (colonic) and systemic inflammation in patients being screened for colorectal cancer?

RQ5- Do local inflammatory infiltrates differ between normal tissue, high and low grade dysplastic adenomas and colorectal adenocarcinomas?

D. Study Design and Measurements

Participants will be recruited via the Scottish Bowel Screening Programme. We propose to send out:

a) a food frequency questionnaire (FFQ) and

b) a calprotectin kit for collection of a faecal sample along with the information and bowel preparation that is already posted to patients prior to their colonoscopy

Informed consent will be sought by providing information in advance of the colonoscopy and by discussing it at the time of colonoscopy. Participants will have from the time they receive these documents by post until their arrival at the endoscopy unit to consider their decision. The consent form will be signed by the member of nursing staff looking after the patient when given to them and any questioned answered.

Prior to colonoscopy and sedation patients will have height and weight measurements taken in the department. If the facilities are not available to carry out these measurements then the patient will be asked to self-report these measurements.

During cannulation, which is required for administration of sedative medications during colonoscopy, we propose to remove a sample of blood for analysis of CRP and IL-6 levels, a differential white cell count, an albumin level and a serum calprotectin. Verbal consent will be sought.

Consent will also be sought to use any surplus tissue removed at colonoscopy (either polypectomy or biopsy) for analysis of local inflammatory infiltrates (including immunohistochemical analysis of immune cell infiltrates and of the IL-6 receptor pathway). Cell surface antigens to be evaluated include CD4+, CD8+, CD68+, CD45RO+ and FOXP3+.

- Hypothesis:
  -Of those attending for colonoscopy via the bowel screening programme, patients with colorectal adenomas or colorectal adenocarcinomas have a different dietary pattern (e.g. less consumption of polyphenols and fibre) than those with a normal colon.

  - Of those attending for colonoscopy via the bowel screening programme, patients with colorectal adenomas or colorectal adenocarcinomas demonstrate a greater inflammatory response as measured by a) faecal calprotectin and b) serum CRP levels and serum calprotectin and c) the white cell count/albumin level.

  -Inflammatory infiltrates differ between normal tissue, high and low grade dysplastic adenomas and adenocarcinomas diagnosed in those attending for colonoscopy via the bowel screening programme.

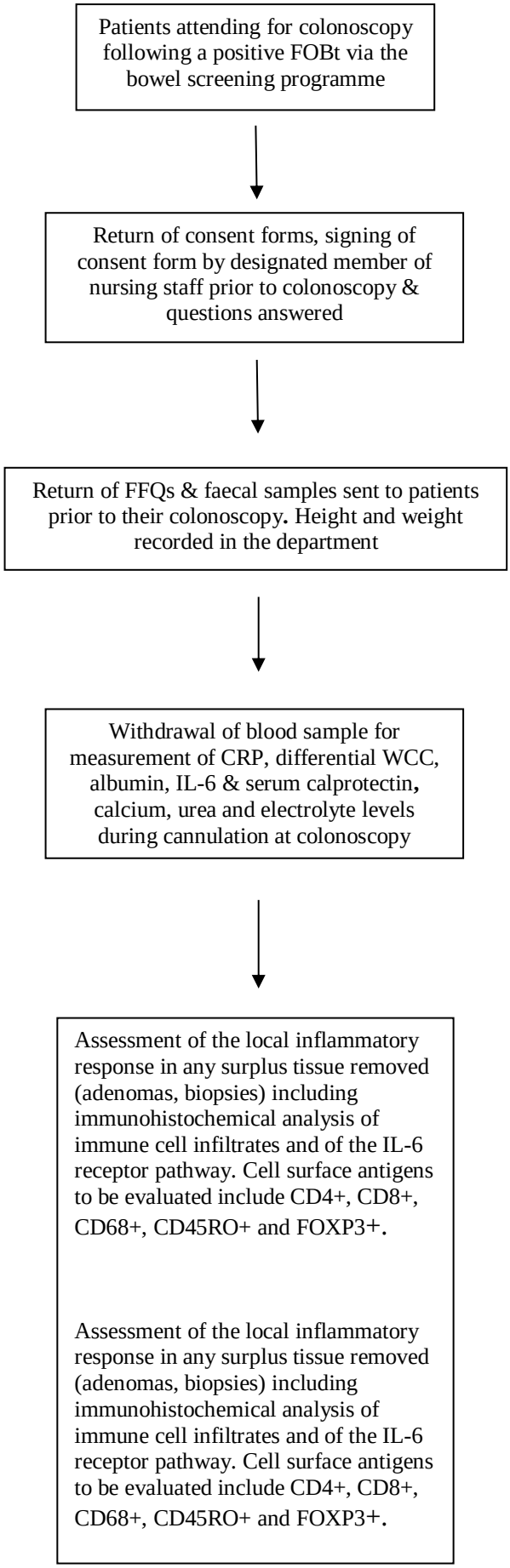
- Sample size: Based on our sample size calculation, 298 participants are required. However, approximately 1000 patients attend for a screening colonoscopy per year (5 lists per week across the sites, 4 patients per list over 50 working weeks) and we aim to capture as many of these as possible. The primary aim of this study is to ascertain whether the diagnosis of colorectal neoplasia is influenced by dietary level of polyphenol consumption. In the Scottish population, advanced neoplasia rates are 40%. Based on a baseline rate of 40% with a power of 80% and a significance level of 95% and aiming to detect a 15% reduction in advanced neoplasia rates, a sample size of 298 is required.

  We also wish to ascertain whether a diagnosis of colorectal cancer is influenced by diet ary polyphenol consumption.The detection rate within this group of patients is currentl y 10%.

  Based on a baseline rate of 10% with a power of 80% and a significance level of 95% a nd aiming to detect a 5% reduction in cancer rates, a sample size of 864 is required. Th is number should be sufficient to generate valid hypotheses and also to accurately carry out sample size calculations for future hypothesis led studies. In addition, this would al low for subgroup aanalysis beyond the primary outcome measure.

- Statistical analysis: Results will be expressed as median/mean (range) and analysed using non-parametric statistics. Statistical significance shall be set at $p < 0.05$. Correlations between categorical variables will be assessed using chi squared tests for linear trends. The Mann Whitney test will be used for 2 group comparisons of nonparametric data. Multivariate analysis will be utilised to assess relationships in the presence of multiple associated factors. Kaplan Meier survival plots will be utilised to establish the significance of specific host and tumour prognostic factors identified through analysis.

Analysis will be performed using SPSS software (SPSS Inc., Chicago, IL, USA).


Patients attending for colonoscopy following a positive FOBt via the bowel screening programme
Return of consent forms, signing of consent form by designated member of nursing staff prior to colonoscopy & questions answered
Return of FFQs & faecal samples sent to patients prior to their colonoscopy. Height and weight recorded in the department
Withdrawal of blood sample for measurement of CRP, differential WCC, albumin, IL-6 & serum calprotectin, calcium, urea and electrolyte levels during cannulation at colonoscopy
Assessment of the local inflammatory response in any surplus tissue removed (adenomas, biopsies) including immunohistochemical analysis of immune cell infiltrates and of the IL-6 receptor pathway. Cell surface antigens to be evaluated include CD4+, CD8+, CD68+, CD45RO+ and FOXP3+.
Assessment of the local inflammatory response in any surplus tissue removed (adenomas, biopsies) including immunohistochemical analysis of immune cell infiltrates and of the IL-6 receptor pathway. Cell surface antigens to be evaluated include CD4+, CD8+, CD68+, CD45RO+ and FOXP3+.

E. Sample and data analysis

- Faecal calprotectin samples will be sent to the NHS laboratory to be analysed, as will the blood samples.

- Surplus tissue samples will undergo immunohistochemical analysis for immune cell infiltrates, IL-6 receptor pathways and the cell surface antigens to be evaluated include CD4+. CD8+, CD68+, CD45RO+ and FOXP3+.

- Data will be analysed by the named researchers at the Academic Unit of Colorectal Surgery, 2nd Floor, New Lister Building, Glasgow Royal Infirmary. All data will be stored on University servers or on an encrypted hard drive. Paper based data will be archived in lockable filing cabinets. Access will be by Mr Roxburgh, Dr Combet or Miss Little, Mr Di Rollo and Miss Ross.

-Data analysis

-The results may be disseminated through peer reviewed scientific journals and conference presentations and will be included in a thesis which will be written in order to obtain a higher degree.

Patients will not individually be notified of the results unless any results are grossly abnormal, in which case the patient will be informed along with their General Practitioner in order to determine if further investigation is required. Should the patients wish copies of any publications or thesis, this can be provided on request as well as a lay summary of findings.

F. Duration of recruitment

1 year

G. Subjects and assessment procedures

Inclusion criteria

- Patients attending following positive FOBt via the Scottish Bowel Screening Programme

- Aged 50-74 years

- Either male or female.

Exclusion criteria

-All of those with a positive bowel screening test will be included.

Location:

- Those who attend for colonoscopy at *Glasgow Royal Infirmary*, *Stobhill Hospital Gartnavel General Hospital and The New Victoria Hospital* via the Scottish Bowel Screening Programme

H.Ethics

This project is being reviewed by the NHS GG&C Research and Development department with the support of the NHS Colorectal Cancer Screening Programme.

I. Funding

This work will performed by the NHS laboratory as the tests are clinically indicated in this group of patients. However, should extra funding be required, this shall be covered by the Academic Unit of Colorectal Surgery, Glasgow Royal Infirmary.