Evaluating the role of angiogenesis in disease behaviour and therapy response in ulcerative colitis

Submitted for the degree of Medical Doctorate in clinical medicine

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Thesis Summary:

Ulcerative colitis (UC) is a chronic inflammatory condition with a significant impact on quality of life. In this thesis two small molecule anti-angiogenic and anti-inflammatory agents were used to treat tissue cultured media (TCM) from UC colonic explants. Targeting angiogenesis may provide a novel therapeutic pathway in the management of UC.

In chapter 1 the role of angiogenesis in the pathogenesis of UC was reviewed. Angiogenesis is a process that in normal circumstances is tightly regulated, dysfunction in this regulation can cause angiogenesis to be continuously switched on and pathological in nature. Angiogenesis and chronic inflammation are inter-related. Despite robust evidence of a significant relationship between pathological angiogenesis and active UC there have been very few studies that have attempted to therapeutically target this pathway.

The two novel small molecule drugs used in this thesis were derived from parent drug quininib. Quininib was screened from a library of over 50,000 compounds and found to display significant anti-angiogenic activity in zebrafish. Quininib analogues were formed, from which P3 and Q8 were two lead compounds. P3 out-performed parent drug quininib at inhibition of angiogenesis in an *in vivo* zebrafish model. No gross morphological anomalies or toxicities were noted. In an isogenic radioresistant oesophageal adenocarcinoma model, P3 was found to reduce inflammatory analyte expression. Q8 inhibited angiogenesis in zebrafish and had a favourable side effect profile. Q8 displayed vascular endothelial growth factor (VEGF)-independent anti-

angiogenic effects in a colorectal cancer (CRC) explant model. Both these agents display potent anti-angiogenic and anti-inflammatory characteristics and as a result were labelled lead compounds and earmarked for evaluation in UC.

In chapter 3, clinical characteristics of a UC patient cohort were evaluated. The colonic microenvironment was investigated for angiogenic and inflammatory analyte expression. C reactive protein (CRP) is an acute phase inflammatory marker, in this chapter we showed that it correlated with disease severity. Interestingly, we also found that an elevated serum CRP correlated strongly with increased tumor necrosis factor (TNF)-α in the colonic microenvironment. This is the first time this has been demonstrated and further strengthens the role for CRP to assess disease severity and therapy response in UC.

In chapter 4 both drugs were evaluated for their ability to suppress the release of key inflammatory and angiogenic analytes. Both P3 and Q8 led to a significant reduction in VEGF-A. VEGF-A is the most potent driver of angiogenesis and has been shown to be associated with increased disease activity in IBD. P3 also inhibited VEGF-C and placental growth factor (PIGF) when compared with control. Both P3 and Q8 had a significant anti-inflammatory effect. Both agents suppressed the secretion of TNF-α, interleukin (IL)-2, IL-8, IL-12p70 and IL-13. TNF-α is an important therapeutic target in UC. This study provides evidence that P3 and Q8 may have a role in the management of UC. Both molecules display strong anti-inflammatory and anti-angiogenic effects in the UC colonic microenvironment. These findings are consistent with previous data generated in zebrafish, CRC and oesophageal cancer explants.

Dendritic cells (DCs) are key cells in adaptive immunity. In chapter 5 the effects of TCM on DC maturation was evaluated and the impact of P3 and Q8 studied. When evaluating the effect of each drug on DC maturation, P3 was associated with increased expression of maturation markers CD54 and PD-L1, when compared with control. Other markers of maturation were similar in each group, suggesting that neither drug significantly impacts on DC function.

Acknowledgements

Abbreviations

5-ASA	5-Aminosalicylic acid
5-LO	5-lipoxygenase
AA	Arachidonic acid
AML	Acute myeloid leukaemia
AMNCH	Adelaide and Meath Hospital Dublin
ANG	Angiopoietin
AS	Ankylosing spondylosis
BCA	Bicinchoninic acid
bFGF	Basic fibroblast growth factor
CAM	Cellular adhesion molecules
CD	Crohn's disease
CML	Chronic myeloid leukaemia
COPD	Chronic obstructive pulmonary disease
CRC	Colorectal cancer
CREB	cAMP response element binding
CRP	C reactive protein
CSF	Colony stimulating factor
CysLT	Cysteinyl leukotriene receptor
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPO	Data protection officer
DSS	Dextran sulfate sodium
DVT	Deep vein thrombosis
ECAR	Extracellular acidification rate
EC	Endothelial cell
ECL	Electrochemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELAM-1	Endothelial leukocyte adhesion molecule 1
ELISA	Enzyme-linked immunosorbent assay
EN	Erythema nodosum
EPR	Electronic patient record
ER	Endoplasmic reticulum
ESR	Erythrocyte sedimentation rate
FACS	Fluorescence-activated cell sorting
FBC	Full blood count
FC	Faecal calprotectin
GDPR	General Data Protection Regulation
GI	Gastrointestinal
GIST	Gastrointestinal stromal tumor

GRO	Growth regulated oncogene
Gy	Gray
Hb	Haemoglobin
HCC	Hepatocellular carcinoma
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factor
HRR	Health research regulation
IBD	Inflammatory bowel disease
ICAM	Intercellular adhesion molecule
IgA	Immunoglobulin A
IGF	Insulin like growth factor
IL	Interleukin
INF-γ	Interferon-gamma
IV	Intravenous
LPS	Escherichia coli Lipopolysaccharide
M	Macrophage
MACS	Magnetic-activated cell sorting
MADCAM	Mucosal addressin cellular molecule
MAPK	Mitogen-activated protein kinase
MC	Mesenchymal cell
MCP	Monocyte chemoattractant protein
MGST2	Microsomal glutathione S-transferase 2
MHC	Major histocompatibility complex
MI	Myocardial infarction
MLN	Mesenteric lymph node
MMP	Matrix metalloproteinase
MSD	Meso scale discovery
NADPH	Nicotinamide adenine dinucleotide phosphate
NBI	Narrow band imaging
NF-kB	Nuclear factor kappa B
NK T Cell	Natural killer T cell
NO	Nitric oxide
NOX4	NADPH oxidase 4
OAC	Oesophageal adenocarcinoma
OCR	Oxygen consumption rate
PAI	Plasminogen activator inhibitor
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factors
PDGFR	Platelet-derived growth factor receptor
PDL-1	Programmed death ligand-1
PE	Pulmonary embolism
PECAM	Platelet endothelial cell adhesion molecule

PG	Pyoderma gangrenosum
PGF	Placental growth factor
PI3K	Phosphoinositide 3-kinase
PMS	Partial mayo score
PRES	Posterior reversible encephalopathy syndrome
PSC	Primary sclerosing cholangitis
RA	Rheumatoid arthritis
ROS	Reactive oxidative species
RPMI	Roswell park memorial institute medium
RT	Room temperature
SJH	St James's Hospital
SMC	Smooth muscle cell
SpA	Seronegative spondyloarthropathy
STAT	Signal transducer and activator of transcription
TCM	Tissue cultured media
TGF-β	Transforming growth factor beta
Th	T-helper cell
Tie	Angiopoietin receptor
TNBS	Trinitrobenzene sulphonic acid
TNF-α	Tumor necrosis factor alpha
TPMT	Thiopurine methyltransferase
TREG	Regulatory T cell
TSP-1	Thrombospondin-1
UC	Ulcerative Colitis
UV	Ultraviolet
VCAM	Vascular cell adhesion molecule
VEGF	Vascular Endothelial Growth Factor
WCC	White cell count

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Chapter One: Introduction

1.1 Introduction

In this thesis the role of angiogenesis and inflammation in ulcerative colitis (UC) was evaluated. The relationship between clinical characteristics and angiogenic molecule expression was explored. Novel patented small molecule drugs were used to therapeutically target the angiogenic cascade.

Angiogenesis is the growth of new blood vessels from pre-existing vascular structures. It is a tightly regulated complex physiological process that involves multiple angiogenic mediators. Imbalance between these mediators leads to pathological angiogenesis (1). Angiogenesis and chronic inflammation are closely related processes that perpetuate each other. The altered colonic microenvironment seen in patients with ulcerative colitis drive pathological angiogenesis, persistent inflammatory damage lead to dysregulated and uncontrolled release of angiogenic molecules (2). Vascular involvement has been a known factor in UC for over 50 years (3). Studies have shown that angiogenic and lymphangiogenic factors have a significant role in UC. These factors are increased in patients with UC and correlate with disease activity (4). This thesis will further characterise the angiogenic and metabolic microenvironment in UC.

To date different angiogenic mediators have been evaluated and their role in UC studied. Vascular Endothelial Growth Factor (VEGF) is the main angiogenic molecule. VEGF is shown to be significantly higher in patients with clinical and endoscopic evidence of disease activity (4, 5). Angiopoietins are important angiogenic mediators, serum angiopoietin 1 and 2 are predictive of disease severity in inflammatory bowel

disease (IBD) and could have a role as a non-invasive biomarker. Platelet-derived growth factors (PDGF), hypoxia-inducible factor (HIF), matrix metalloproteinase (MMP) and cellular adhesion molecules (CAM) have also been studied and are found at increased levels in active IBD (6, 7).

Although there is evidence highlighting the importance of angiogenesis in the pathogenesis of UC, there have been very few successful therapies developed directly inhibiting these pathways. In one report, *Danese et al*. showed that in two different animal models targeting of the VEGFR/VEGF-C pathway displayed marked protection against the development of acute and chronic colitis (8). Evaluation of therapeutics in this area requires further study. This project will evaluate the anti-angiogenic and anti-inflammatory effect that two patented therapies have on *ex vivo* colonic tissue explants from patients with UC.

The role of angiogenesis and energy metabolism has been comprehensively explored in other disease areas, particularly oncology. In this field, novel therapies have successfully targeted angiogenic mediators. Bevacizumab is used in the treatment of colorectal cancer (CRC). It is a monoclonal antibody which inhibits VEGF A signalling. When used as a combination therapy in the treatment of CRC it has been shown to convey a significantly improved survival (9). Sorafenib inhibits VEGFR 1, 2 and 3. It has been used as a therapy in the treatment of advanced hepatocellular carcinoma and improved median survival by 3 months (10). The outputs of this novel project will help position the therapeutic targeting of these pathways in UC.

1.2 Ulcerative colitis

1.2.1 Etiology of Ulcerative colitis

Inflammatory bowel disease (IBD) is a chronic condition of the digestive tract. The two main types are UC and Crohn's disease (CD) (11). IBD is a chronic condition with an etiology that is incompletely understood. It is thought to arise due to a dysregulated host immune response occurring due to environmental triggers, including the intestinal microbiota, in a genetically susceptible host. IBD is characterised by a relapsing and remitting course of varying severity. UC was first described by two British physicians in 1875, Moxon and Wilks distinguished the condition as a separate clinical entity to infectious diarrhoeal diseases. It wasn't until 1932 that CD was described, up to that point most small bowel pathology was labelled as intestinal tuberculosis. In both cases no infectious pathogen could be identified and the possibility of an autoimmune etiology was raised. In the subsequent years with the advent of the fibreoptic colonoscope, the diagnosis of IBD was revolutionised. Major scientific break throughs in immunology, pharmacology, genetics and histopathology led to increased diagnosis and improved therapeutic options.

1.2.2 Epidemiology

IBD has become an increasingly common diagnosis and accounts for a significant proportion of a gastroenterologist's patient cohort. Globally there is a prevalence of 84.3 per 100, 000 population, however this is significantly higher in the western nations. In North America, were prevalence is highest, there are 422 patients per 100, 000 population, compared with the Caribbean with 6.7 per 100, 000 population

(Figure 1.1). From 1990 to 2017 the number of patients with IBD increased from 3.7

million to over 6.8 million (12). This likely reflects increasing prevalence, but also

improved diagnostics and earlier detection. Multiple studies have shown high socio-

economic status to be associated with increased prevalence of IBD, this may be

attributable to the differences in lifestyle and diet amongst this population (13, 14).

Smoking appears to be protective against UC development, however the converse is

true for CD (15). A multitude of environmental factors have been implicated in IBD

development (chewing gum, toothpaste, etc) as well as early exposures

(gastroenteritis, increased hygiene, antibiotics, bottle feeding) with little robust

evidence to support this. In a large Scandinavian study patients with UC were less

likely to have had an appendicectomy than the general population (16). The reason for

this is unknown, appendicectomy has been tried as a therapeutic and preventative

measure in UC on patients without appendicitis with some success (17).
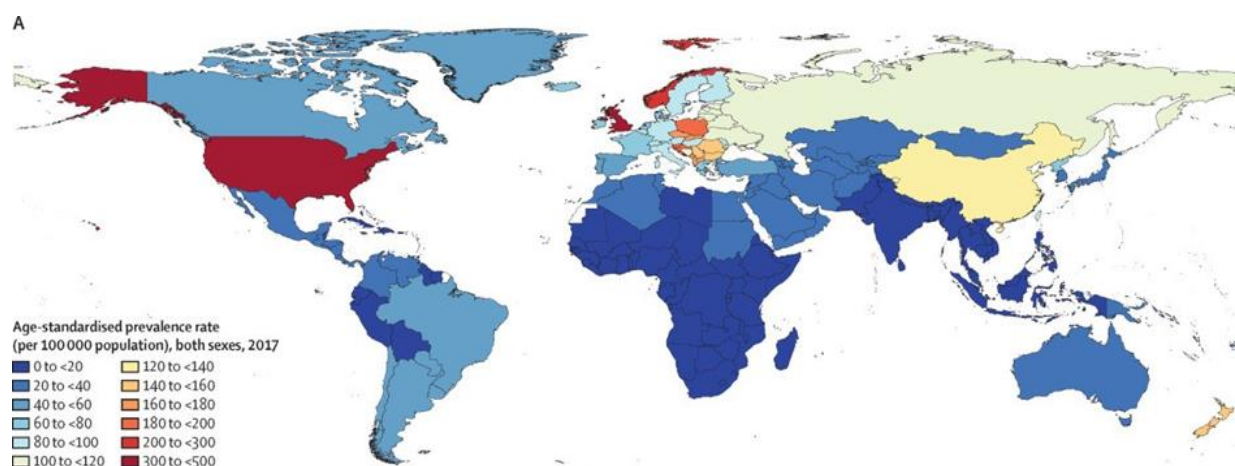
Figure 1.1 Global IBD prevalence.
Increased prevalence of IBD in northwest Europe and the USA, taken from Alatab et al, 2020 (12).

1.2.3 Signs and symptoms of ulcerative colitis

UC is characterised by a relapsing and remitting course of varying severity. The condition has a significant impact on patient's quality of life; it is associated with a high symptom burden and considerable risk of disability. 90% of patients with UC present with bloody diarrhoea. Urgency in defecation and abdominal pain are also common symptoms (18). In some cases, patients are asymptomatic and the diagnosis of UC is picked up incidentally on colonoscopy. There is an increased risk of CRC developing in patients with UC and colonic CD. That risk is associated with disease duration, distribution and severity (19). Other factors like a family history of colorectal cancer or a personal history of primary sclerosing cholangitis (PSC) will also increase the risk. It is hoped that improved treatments and surveillance programs will reduce the incidence of CRC amongst this population.

Both UC and CD are associated with extraintestinal manifestations, most commonly affecting the joints, skin or eyes. IBD associated arthropathy is the most common extraintestinal condition. This can involve both the axial and peripheral joints. 10% of IBD patients experience eye problems. Uveitis is one of the most common. Inflammation of the uvea can cause blurred vision, photosensitivity and pain. The skin is frequently involved in IBD patients. The most common conditions seen are erythema nodosum (EN) and pyoderma gangrenosum (PG).

1.2.4 Diagnosing ulcerative colitis

The diagnosis of UC is based on clinical findings, biochemical/stool analysis, colonoscopy, histology and cross-sectional imaging. It requires infection be excluded and chronicity be established. Biochemical assessment includes full blood count (FBC), urea and electrolytes, liver enzymes, CRP and albumin. Although not diagnostic, the biochemical profile can support the diagnosis and give an indication of disease severity. CRP and white cell count (WCC) may be raised with active inflammation and associated with more severe disease activity. Albumin is inversely related to inflammation and may be low. Some patients have an iron deficiency anaemia due to rectal blood loss. Stool analysis should be carried out in all patients with suspected UC. Stool should be sent for faecal calprotectin, microscopy, culture and sensitivity. Faecal calprotectin (FC) is an accurate marker of intestinal inflammation, it is a very useful tool for evaluating whether patients need to have a colonoscopy. If the clinical suspicion is low then a normal faecal calprotectin indicates a diagnosis of UC is unlikely (20). The common microbial pathogens should be excluded with specific testing for clostridium difficile toxin. Clostridium difficile can cause a pseudomembranous colitis that may be mistaken for UC, a thorough history including any recent antibiotics is essential as well as stool testing.

In UC there is continuous circumferential mucosal inflammation (Figure 1.2), it affects variable extents of the colon with, very often, a clear demarcation between the diseased segment and normal bowel (21). Pseudopolyps may be noted during colonoscopy, these are small masses of hyperplastic granulation tissue formed due to repeated colonic inflammation and regeneration. Colonoscopy is also important to

exclude CRC. Where possible ileocolonoscopy should be carried out in all newly

diagnosed patients, if there is evidence of severe disease activity it is reasonable to

perform a flexible sigmoidoscopy as full colonoscopy has increased risk of perforation.

Biopsies should be taken for histological assessment. Basal plasmacytosis on

histological examination is a high predictor of UC. Other features include architectural

changes, crypt destruction and ulceration.
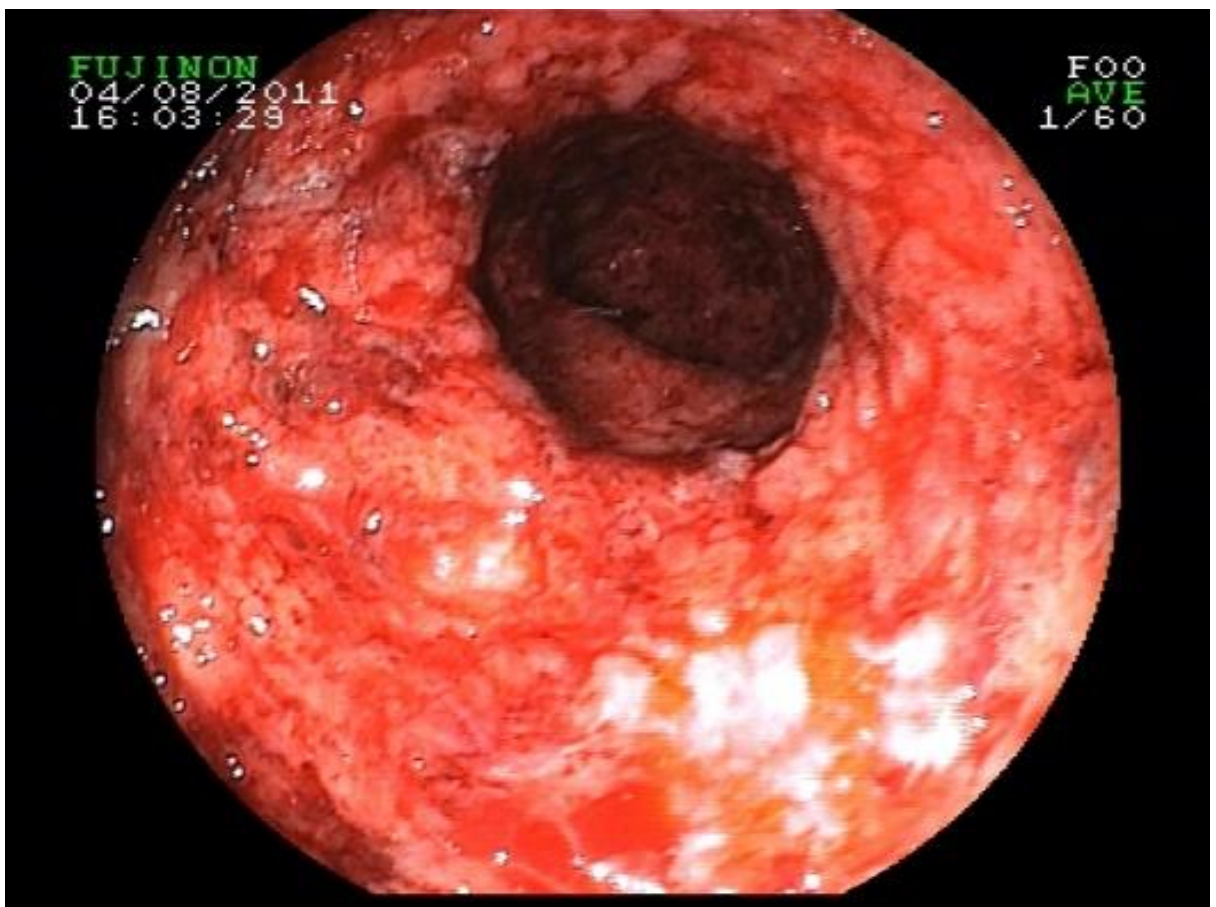
Figure 1.2 Active UC.
Image taken at colonoscopy demonstrating circumferentially inflamed haemorrhagic
mucosa consistent with severe active UC.

1.2.5 Management of ulcerative colitis

The management of UC is dependent on the patient's symptom profile and disease severity. In some cases, treatment can be tailored in a step wise fashion until adequate control is obtained (step-up approach). When symptoms are severe and there is significant disease activity a more aggressive approach is required, in these instances potent immunosuppressive medications or surgery may be necessary as first line treatments (step-down approach). In recent years there has been a shift in the aims of therapy. Control of patient symptoms is understandably an important target but there has been increased focus on achieving mucosal healing also. Early mucosal healing has been shown to be associated with improved clinical outcomes and reduced risk of colectomy (22).

Until recently the mainstay of treatment for UC were 5-aminosalicylates, corticosteroids and thiopurines. Patients who were refractory to these therapies required surgical intervention. Of the 5-aminosalicylate drugs, mesalamine, is the most widely used in the management of UC. Mesalamine can be delivered orally or rectally as a suppository or enema. It has been shown to be effective at inducing and maintaining remission in mild to moderate UC (23). Compared with other therapy options it has a very favourable side effect profile. Corticosteroids are used to treat flares and induce remission. Due to side effects (osteoporosis, hypertension, weight gain) associated with long term use they are not given as maintenance therapy. Steroids are often given over an 8-week course with a slowly tapering dose to avoid adrenal insufficiency. Azathioprine is an immunosuppressive drug; it is converted to its active metabolite by thiopurine methyltransferase (TPMT). It inhibits purine synthesis;

this reduces white blood cell production leading to immunosuppression. A proportion of the population do not produce TPMT at adequate levels and as a result are at risk of severe side effects with azathioprine. Azathioprine is a carcinogen and has been linked with the development of skin cancers, lymphoma, mesenchymal and hepatobiliary tumors (24). The carcinogenic risk increases with age. It can cause bone marrow suppression and pancreatitis. Despite its side effects azathioprine is an effective treatment for UC. It is not used as an induction agent as it can take weeks to exert a clinical effect, but works well as a maintenance therapy (25).

TNF -α was identified as an important cytokine driving inflammation in UC (26). It has been targeted effectively with Anti-TNF agents. In 2005 Infliximab a chimeric monoclonal antibody was shown to be effective in induction and maintenance of clinical remission in patients with UC (27). It is licensed for use in patients with moderate to severely active UC who have had inadequate response to conventional therapy. Infliximab is delivered via IV infusion. Patients receive an induction dose at week 0, a further dose at week 2 and 6, then every 8 weeks thereafter. It is possible to monitor drug and antibody levels to ensure adequate dose is being delivered. Although the role of drug monitoring has not been fully established, detectable drug levels are predictive of clinical remission and endoscopic healing, while an undetectable drug level is associated with increased risk of colectomy (28). Adalimumab is another agent that targets TNF-α, it is a human monoclonal antibody which reduces the risk of rejection. It is delivered via subcutaneous (SC) injection making it a more convenient option. The drug is given at week 0, week 1 and every 2 weeks thereafter. It has been shown in a double blinded placebo controlled trial to be

a safe and effective agent at inducing and maintaining remission in moderate to severely active UC (29). Biologic agents have revolutionised the management of UC, however there are significant side effects. Careful history taking is essential, prior exposure to TB, HIV, HBV or other infections need to be out ruled before starting therapy. Side effects of anti-TNF agents include opportunistic infections, lymphoproliferative disorders and worsening of heart failure. An improved understanding of the immunopathology of UC has helped in development of more targeted therapies (30). Lymphocyte trafficking antagonists have been studied. Among these, vedolizumab is approved for use in both UC and CD. Vedolizumab, a monoclonal antibody to alpha4 beta 7 integrin, has demonstrated efficacy as a therapy for UC and CD (31). More recently tofacitinib has been approved for use in UC. This novel janus kinase (JAK) inhibitor is effective in the treatment of UC (32). Ustekinumab targets the p40 subunit of IL-12 and IL-23. It was found to be effective at achieving and maintaining remission in UC (33). Many other novel pathways are being targeted with new agents (Figure 1.3). These include anti-mucosal vascular addressin cell adhesion molecule 1 agents, anti-interleukin monoclonal antibody, an anti-SMAD7 antisense oligonucleotide, and a sphingosine-1-phosphate receptor-1 selective agonist (30). Some of these target pathways influence the angiogenic microenvironment, however none directly target it. The treatment options available for patients with UC are likely to continue to expand.

In some cases, surgery is required in the management of UC. In patients with refractory bleeding, failure of medical therapy, colonic perforation, toxic megacolon or dysplasia/CRC, surgery may be warranted. The potential that surgery may be required

should be discussed with patients as early as possible with prompt referral to a colorectal surgeon. Patients should be seen by a stoma care nurse. If possible, steroids should be weaned prior to surgery. In the elective setting a pan-proctocolectomy is the surgery of choice and is curative in UC. Ileal pouch anal anastomosis is an attractive option as it will restore GI continuity and avoid the need for a permanent stoma. In the acute setting the rectum is left in-situ and often managed later when the inflammation has settled and the patient is well. Although surgery is daunting and often seen as a failure of medical therapy it is the only option which can provide a cure and the majority of patients have a significant improvement in quality of life following surgery.
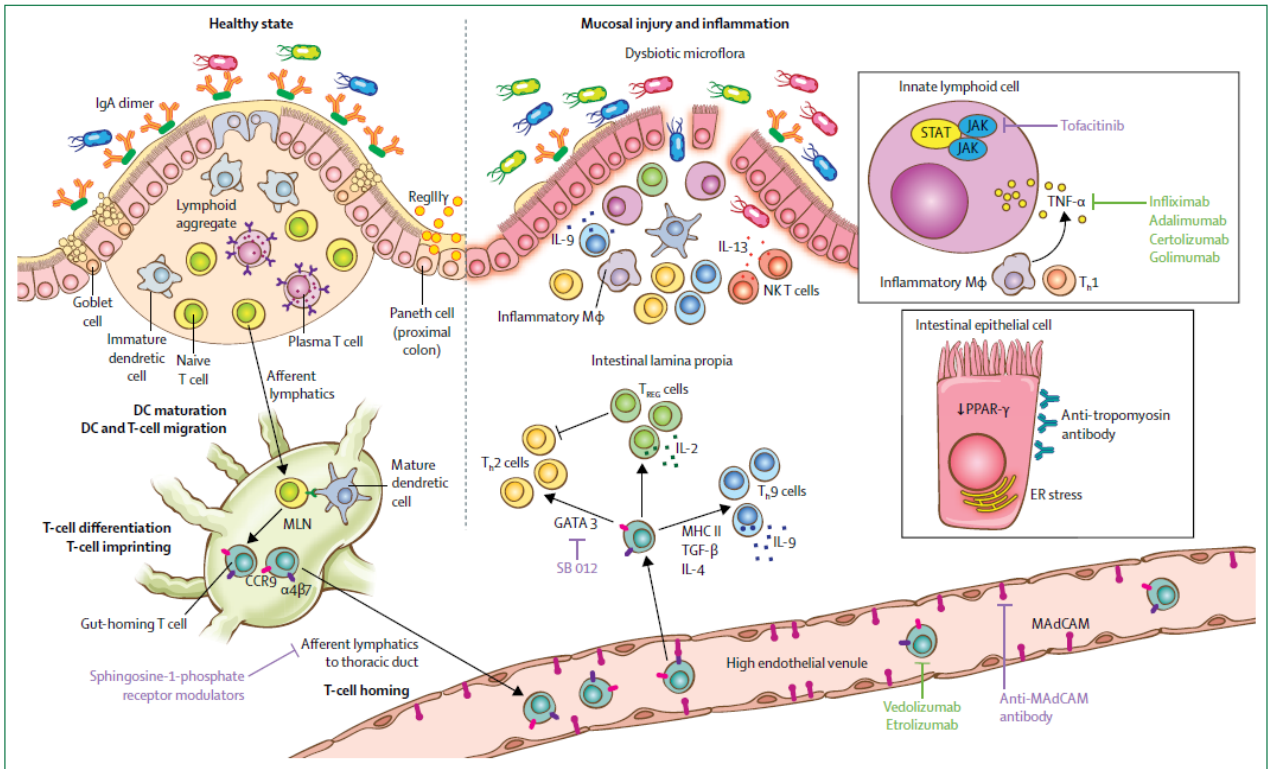
Figure 1.3 UC drug targets.

In a healthy state a barrier is maintained across tight junctions (left). With mucosal injury, as in UC, there is breakdown of the barriers. Current therapies in UC target numerous cytokines in the inflammatory cascade (right). IgA, immunoglobulin A; DC, dendritic cell; IL, interleukin; MAdCAM, mucosal addressin cell associated molecule; TREG, regulatory T cell; Th, T-helper cell; IFN, interferon; TGF, transforming growth factor; M, macrophage; ER, endoplasmic reticulum; NK T Cell, natural killer T cell; MHC, major histocompatibility complex; MLN, mesenteric lymph node. Adapted from Ungaro et al (34).

1.3 Angiogenesis

1.3.1 Importance of angiogenesis

Angiogenesis is an important physiological process by which new blood vessels form from the extension and elaboration of pre-existing ones. The initial blood vessels are formed at the embryonic stage through a process called vasculogenesis, subsequent to embryonic development angiogenesis is responsible for blood vessel growth (35). The process is tightly regulated by stimulating and inhibiting factors. Angiogenesis plays an essential role in tissue repair, foetal development and the female reproductive cycle. Unregulated angiogenesis is an important factor driving several pathological conditions. In normal physiological states, angiogenesis subsides and is turned off once the task is complete. In pathology, in particular in malignancy, angiogenesis is not self-limiting and continues indefinitely (36). Pathological angiogenesis is a hallmark of various ischaemic, inflammatory and neoplastic diseases. Research in this area is leading to the discovery of a significant number of anti- and pro-angiogenic agents (37). Angiogenesis has emerged as an important factor in the growth of tumors; its inhibition is a cornerstone in the treatment of cancer. The importance of targeting angiogenesis extends beyond cancer biology and is an integral component of autoimmune and chronic inflammatory diseases such as rheumatoid arthritis, atherosclerosis, diabetic retinopathy, peptic ulcers, psoriasis and Alzheimer's disease (1).

Angiogenesis occurs over several stages (Figure 1.4). Tissue damage or hypoxia lead to pro-angiogenic stimulation. The release of Nitric Oxide (NO) and VEGF causes blood

vessel dilatation and increased permeability. Thereafter other factors, particularly

transforming growth factor beta (TGF-β), MMPs and Angiopoietin-1 (Ang-1), promote

degradation of the extracellular matrix, basement membrane and pericytes, followed

by remodelling of the extracellular matrix. Proliferation and migration of endothelial

cells is stimulated by VEGF, placental growth factor (PGFs), epidermal growth factor

(EGFs) and integrins. The mesenchymal cells differentiate into smooth muscle cells and

pericytes that stabilise and control the new endothelial cells.
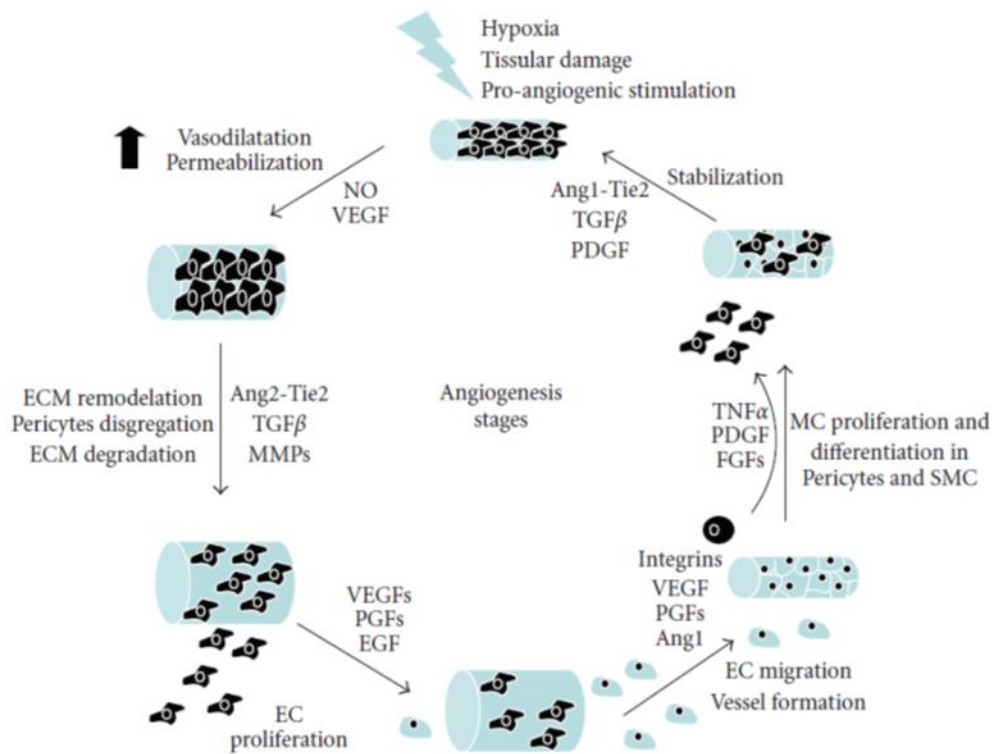
Figure 1.4 *Stages of angiogenesis.*
Hypoxia and tissue damage lead to vessel vasodilation. Extracorporeal membrane
(ECM) remodelling and endothelial cell (EC) proliferation driven by VEGF. EC migration
and vessel formation is followed by differentiation and stabilisation. MC, mesenchymal
cell; SMC, smooth muscle cell Taken from Sanz-Cameno et al (38).

1.3.2 Angiogenesis and inflammation

Angiogenesis and inflammation were initially thought to be very separate entities however research has shown significant overlap. Both processes augment cellular infiltration and proliferation, they have overlapping roles in regulation of cytokines and growth factors (39). Although chronic inflammation invariably results in angiogenesis, angiogenesis can occur without inflammation. Prolonged and uncontrolled angiogenesis is a hallmark of chronic inflammatory disorders. Mast cells, macrophages, monocytes and other leukocytes release a vast array of angiogenic factors including VEGF, platelet-derived growth factor (PDGF), TGF-β, basic fibroblast growth factor (bFGF) and many more. Red cells contain proteinases that degrade barriers for migrating vascular cells and activate some of the growth factors from the extracellular matrix (40). Both angiogenesis and inflammation are exacerbated by the release of these molecules. In UC, mucosal inflammation is characterised by vasodilation and increased permeability that continuously drives release of angiogenic molecules.

Along with inflammatory cytokines, the conditions caused by inflammation can also drive angiogenesis. Plasma extravasation and tissue hypoxia occur in inflamed tissue resulting in angiogenesis. Hypoxia causes release of VEGF triggering angiogenesis in an attempt to oxygenate the inflamed tissue.

1.4 The role of angiogenesis and the vasculature in ulcerative colitis

Angiogenesis may be physiological or pathological. Colonic inflammation is followed by physiological angiogenesis; however, chronic ongoing damage can lead to abnormal pro- and anti-angiogenic marker expression. This can affect vascular cell types and vascular remodelling, leading to impaired or pathological angiogenesis (2). Micro vessels in the chronically inflamed colons of patients with UC have displayed significant alterations in physiology and function when compared with micro vessels from healthy individuals. Functional and morphological abnormalities in the expression of angiogenic markers like VEGF are seen in both IBD and experimental colitis (41, 42). The intestinal microcirculation plays an important role in maintaining mucosal homeostasis (43). The creation of new blood vessels is closely regulated by pro- and anti-angiogenic markers, but in UC angiogenesis is driven by immune response and inflammation.

It has long been hypothesised that the vasculature plays a significant role in UC. On a macrovascular level, this is supported by the colonic distribution of UC and the often very clear demarcation between diseased and healthy colon. A study carried out in 1977 demonstrated increased blood flow to the diseased bowel segment, there was intense hypervascularity associated with clinically severe disease (44). Endoscopic evaluation with narrow-band imaging (45) also point towards increased angiogenesis and vascular involvement in UC (46). Narrow-band imaging (NBI) is an imaging technique employed in endoscopy, light of specific green and blue wavelengths enhance the detail of aspects of the mucosa. It provides increased detail allowing

more accurate description of polyps and tumors. NBI provides excellent quality imaging of the colonic vasculature. In patients with UC, the pro-inflammatory and angiogenic state is associated with a threefold increase in the risk of deep vein thrombosis (DVT) and pulmonary embolism (PE), when compared with the general population (47). Treatment of disease flares also require concomitant thromboembolic prophylaxis. Heparin, used in thromboembolic prophylaxis was previously reported to have a therapeutic effect in UC. The mechanism of action was not fully understood but thought to be related to inhibition of thrombin and factor Xa resulting in microvascular thrombosis (48). Studies have shown that angiogenic and lymphangiogenic factors correlate significantly with clinical, endoscopic and histological disease activity (4).

1.5 Angiogenic molecules

The angiogenic cascade involves complex interactions between multiple molecules, many of which have been studied in IBD (Table 1.1). Vascular endothelial growth factors (VEGF) are a family of potent drivers of angiogenesis. They include VEGF A, B, C, D, E, as well as placental growth factor (PIGF) 1 and 2. All members of the VEGF family have a common homology domain. At the core is a cysteine knot motif, at one end there are eight invariant cysteine residues in intra- and intermolecular disulfide bonds with a short three stranded β-sheet at the other end (49). Each VEGF monomer dimerize in an anti-parallel manner. VEGF drives not only pre-existing blood vessel development but also de novo synthesis (Vasculogenesis). They are important in embryonic development and tissue healing. VEGF is an inducer of angiogenesis and lymphangiogenesis because it is a very specific mitogen acting on the endothelium.

Signal transduction leads to the binding of tyrosine kinase receptors resulting in proliferation of endothelial cells, migration and formation of new vessels (49). Uncontrolled VEGF release can drive inflammation and neoplastic growth (50).

VEGF-A is the most potent growth factor in driving angiogenesis. VEGF-A is a dimeric, disulphide-bound glycoprotein. In healthy individuals, VEGF-A is at its highest levels in adult kidney, heart, lung and adrenal gland. Lower levels are found in the gastric mucosa, liver and spleen. VEGF-A bind tyrosine kinase receptors leading to activation of VEGF receptors, which promote angiogenesis. VEGF-A primarily affect endothelial cells, but also stimulate activity in neuronal cells. VEGF-B discovered in 1995, is found at highest concentrations in adult skeletal muscle, myocardium and pancreatic tissue. In contrast to VEGF-A it plays a less pronounced role in angiogenesis. It is important in maintenance of normal cell function in newly formed endothelial cells. It protects neurons in the cerebral cortex and retina. VEGF-C is most prominently expressed in the heart, ovary, small intestine, placenta and the thyroid gland. It undergoes complex proteolytic maturation leading to multiple processed forms. Its primary function is in lymphangiogenesis. VEGF-C acts on lymphatic endothelial cells promoting growth survival and migration. VEGF-D has a significant role in embryonic angiogenesis and lymphangiogenesis. It appears to play a more subtle role in modulating lymphatic vascular development than VEGF-C (51). PlGF is a key molecule in vasculogenesis and angiogenesis during embryonic development; specifically, it is important in trophoblast growth and differentiation.

Studies have evaluated the role vascular endothelial growth factor (VEGF) in IBD. A report from a paediatric IBD population demonstrated serum VEGF levels to be significantly higher in patients with IBD compared with controls. A significant correlation between VEGF levels and disease activity was also observed (52). This supports the hypothesis that pathological angiogenesis has an important role in perpetuating UC disease activity (53). The angiogenic process is a complex, tightly regulated pathway. Another study looking at VEGF-Ets-1 in IBD, found that angiogenic markers were upregulated in UC but not in CD. VEGF-Ets-1 converts endothelial cells to those of an angiogenic phenotype (54). Within the paediatric IBD population VEGF-A expression was associated with upregulated angiogenesis and inflammation. Mucosal angiogenesis is increased at the margins of the surgical resection where there was limited inflammation present (55). VEGF-C has not been studied in IBD, however stimulation of lymphangiogenesis via VEGF-C was shown to increase intestinal inflammation in experimental colitis. When colitis was induced in mice a significant increase in colonic inflammation, oedema and mucosal damage was seen in those overexpressing VEGF-C (56). This, however, is in direct contrast to a similar study which found that VEGF-C stimulation ameliorated experimental colitis (8). Although to date there are no IBD therapies which directly target VEGF, *Algaba et al.* showed that VEGF levels were significantly reduced in patients with IBD on infliximab or adalimumab (57). *Yi Zhou et al* demonstrated the inflammatory and angiogenic effects of PIGF activation in IBD. They studied human intestinal microvascular endothelial cells and calculated PIGF using western blot analysis and quantitative polymerase chain reaction (PCR). PIGF and its receptor are significantly increased in active IBD (58).

The angiogenic effects of Nitric Oxide (NO) have not been fully elucidated. NO is thought to inhibit apoptosis and, by increasing the expression of VEGF/FGF, enhance endothelial cell proliferation. Angiogenesis is accompanied by vasodilation of the surrounding vessels, NO significantly contributes to the proangiogenic environment by triggering vasodilation. Increased NO levels are associated with higher levels of angiogenesis and in malignancy with tumor progression (59).

Table 1.1 *Angiogenic Molecules*

Table detailing the function of angiogenic molecules, medications that target each molecule and the role in IBD.

Molecule	Function	Antagonist	Role in IBD
VEGF-A	Most potent Angiogenic factor, binds tyrosine kinase receptors leading to activation of VEGF receptors	Bevacizumab	In a paediatric IBD population VEGF-A expression was associated with upregulated angiogenesis and inflammation
VEGF-B	Maintenance of endothelial cell function, fatty acid transportation	None	Unknown
VEGF-C	lymphangiogenesis by promoting growth survival and migration of lymphatic endothelial cells	None	Conflicting studies showing VEGF-C worsens and improves experimental colitis in mice
VEGF-D	Angiogenesis and lymphangiogenesis via VEGFR-2/3 activation on endothelial cells	None	Unknown
PIGF	PIGF is a key molecule in vasculogenesis and angiogenesis during embryonic development	None	Pro-inflammatory/angiogenic effects in IBD via PI3k/Akt activation
TIE-2	Regulated angiogenesis, proliferation, migration, adhesion of endothelial cells	Rebastinib, angiopoietin 2	Tie-2 is elevated in patients with IBD
bFGF	Angiogenesis through endothelial cell proliferation	Sunitinib, Suramin, Thalidomide	Serum levels of bFGF did not differ between IBD and healthy patient cohorts
FLT-1	Angiogenesis through endothelial cell proliferation	None approved (Ribozyme, preclinical)	Upregulated in UC, but down regulated in CD
PDGF	Potent mitogen for mesenchymal cells, including smooth muscle cells, fibroblasts and glial cells	Imatinib, pazopanib and nilotinib	Important inducer of fibrosis
CAMs	Cell growth, apoptosis and contact inhibition	Adalimumab,vedolizumab, Alicaforsen	Responsible for adhesion of leukocytes to endothelial

			cells, upregulated in active IBD. ICAM-1 blockade in pouchitis
NO	Release stimulated by VEGF, inhibits apoptosis and increases endothelial cell proliferation	Arginase	An important mediator of mucosal inflammation and disease severity but its role has not been fully elucidated
MMPs	Tissue remodelling: morphogenesis, angiogenesis and tissue repair	Doxycycline	Upregulated in IBD, but when targeted no significant improvement

VEGF-A (Vascular Endothelial Growth Factor-A), VEGF-B (Vascular Endothelial Growth Factor-B), VEGF-C (Vascular Endothelial Growth Factor-C), VEGF-D (Vascular Endothelial Growth Factor-D), PIGF (Placental Growth Factor), TIE-2 (Angiopoietin Receptor), bFGF (Basic Fibroblast Growth Factor), FLT-1 (Vascular Endothelial Growth Factor Receptor-1), PDGF (Platelet Derived Growth Factor), CAMs (Cellular Adhesion Molecules), NO (Nitric Oxide), MMPs (Matric Metalloproteinases)

Basic fibroblast growth factor (bFGF) has a broad range of functions. It is both a growth factor and signalling protein that is encoded by the fibroblast growth factor (FGF) 2 gene. Its biological roles include cell growth, tissue repair, morphogenesis, and embryonic development. BFGF is present in the basement membrane and is activated following the release of heparin sulfate degrading enzymes. It promotes invasion of the extracellular matrix by sprouting endothelial cells. BFGF is synthesized and secreted by adipocytes. In mouse models, it exerts a cardio-protective effect following injury from myocardial infarction. Overexpression of bFGF was associated with reduced tissue death and improved cardiac function following reperfusion (60). However, bFGF does appear to have a significant role in cancer development and progression through its pro-angiogenic activity. BFGF and VEGF act synergistically to amplify cancer angiogenesis by induction of VEGFR and by direct regulation of VEGF expression. It also exerts an indirect effect by promoting Hypoxia inducible factor-1

(HIF-1) (61). The role of basic fibroblast growth factor was reviewed in a paediatric

population with CD, UC and functional abdominal pain. Although the average levels

did not differ significantly between the 3 groups there was a strong correlation

between disease activity and basic fibroblast level in the CD cohort (62). BFGF and

VEGF levels were reviewed in a cohort of patients with CD treated with infliximab.

They found reduced levels of bFGF and VEGF in patients who had received infliximab,

supporting their hypothesis that anti-TNF therapy down regulates angiogenesis (63).

Angiopoietins are a family of three protein growth factors involved in regulating

angiogenesis. They exert their effect by binding to angiopoietin receptors (Tie-1 and

Tie-2). These tyrosine kinase receptors mediate cell signals by triggering

phosphorylation of key tyrosines which promote cell signalling. When angiopoietin

binds Tie-2 it promotes maturation of pericytes and endothelial cells. They play an

important role in vascular stability and maturation during angiogenesis. A study

looking at angiopoietin and TIE-2 expression in patients with hepatocellular carcinoma

(HCC) showed that increased expression was associated with adverse clinical

outcomes (64). High angiopoietin and Tie-2 levels were associated with increased

tumor diameter and portal vein invasion. Survival in this group was significantly lower

when compared with those who had normal angiopoietin and Tie-2 expression.

Angiogenin levels were measured in serum from 154 IBD patients and in 84 healthy

controls. Patients with IBD had elevated levels of serum angiogenin (65). Angiopoietin-

2 and Tie-2 are important serum markers of angiogenesis. A study of 160 patients with

IBD showed angiopoietin-2 and Tie-2 were significantly elevated compared with

healthy control group (80 patients) (66). A study in rats with dextran sulfate sodium (DSS) colitis showed increased expression of angiopoietin-1, 2, TIE-2 and VEGF (67).

Platelet derived growth factor (PDGF) is stored and released by platelets. It plays an important role in angiogenesis, embryonic development, cell migration and proliferation. Recombinant PDGF has been shown to be beneficial in healing chronic ulcers and to stimulate bone regeneration (68, 69). However no data has been published on its role in UC.

Cellular adhesion molecules (CAMs) are a family of immunoglobulins with a significant role in immune response, inflammation and intracellular signalling. This is achieved via activation of lymphocytes and leukocyte trafficking. CAMs are upregulated during inflammation, particularly when sustained over long periods. Included in the family are Intercellular adhesion molecules (ICAMs), mucosal addressing cellular molecule (MADCAM), platelet endothelial cell adhesion molecule (PECAM), vascular cell adhesion molecule (VCAM) expressed on endothelial cells. The expression of endothelial leukocyte adhesion molecule 1 (ELAM-1) was studied in colonic mucosa of patients with IBD and in healthy controls. ELAM-1 was consistently found at high levels in areas of the colon with active inflammation. It was not found in healthy controls or patients with quiescent IBD. ELAM-1 facilitates leukocyte migration into sites of active inflammation in patients with IBD (70).

Matrix metalloproteinases (MMPs) are a family of enzymes that are responsible for the breakdown of the extracellular matrix proteins during normal growth and tissue turnover. MMPs control cell behaviour through finely tuned proteolytic processing of a wide variety of signalling molecules (71). Immunohistochemistry on frozen sections from patients with IBD suggested MMPs are important in angiogenesis, tissue remodelling and leucocyte extravasation (72). Increased expression of endothelial alpha V beta 3 in patients with IBD compared to healthy control patients supports the hypothesis that angiogenesis is integral in IBD (42).

1.6 Anti-Angiogenic effects of current UC treatments

Some of the therapies commonly used in ulcerative colitis have been shown to have an anti-angiogenic effect. Mesalamine is a 5-aminosalicylic acid drug used for mild to moderate ulcerative colitis. It was approved for use in the 1980s and is a safe and effective maintenance therapy. In experimental colitis, Mesalamine has been shown to restore angiogenic balance. It reduces the expression of angiostatin and endostatin (73). Patients with IBD have significantly elevated VEGF, Ang 1 and Ang 2 levels. Corticosteroids are used to treat disease flares with the aim to induce remission. A study comparing serum angiogenic factors in patients with active UC before and after treatment with corticosteroids found that they temporarily alter levels of angiopoietins, VEGF and Tie 2 (5). However, no correlation was found between clinical UC activity and the serum angiogenic factor levels.

Infliximab indirectly down regulates pathological angiogenesis in patients with IBD. This is achieved by leucocyte trafficking inhibiting production of VEGF-A by fibroblasts, it is hypothesised that this reduces inflammation-driven angiogenesis along the gastrointestinal tract and adds to the clinical efficacy of TNF-α blockade (74).

CAM blockade has been a pathway targeted in the treatment of pouchitis and ulcerative colitis with varying degrees of success. An ileal pouch is an artificial rectum, which is created from ileal tissue in a patient who has undergone colectomy. Pouchitis is inflammation of that ileal pouch, it occurs more commonly when the indication for colectomy was ulcerative colitis. Alicaforsen is a novel therapy approved for use in pouchitis. It is an antisense oligoneucleotide that inhibits production of the ICAM-1 protein. ICAM-1 controls VEGF-A mediated NO activity and has a potent anti-angiogenic effect. The treatment is delivered rectally as a topical enema. It has been shown, in an open label trial, to be effective in pouchitis and is currently being evaluated in a multi-centre phase III clinical trial. In patients with UC, it is significantly more effective than placebo when given as an enema for distal colitis. However when compared to mesalamine therapy there was no difference in outcomes (75). Vedolizumab also targets cellular adhesion molecules in particular MADCAM-1, which exerts its effect through leucocyte trafficking. It is a monoclonal antibody that binds integrin α4β7 resulting in a gut specific anti-inflammatory effect. Vedolizumab is given as an intravenous infusion, it has been shown to be an effective and safe drug in the management of CD and UC in a number of phase 3 clinical trials (GEMINI I, II and III).

Ontamalimab has a similar mechanism of action to vedolizumab; it is a human IgG2 monoclonal antibody that targets MAdCAM-1. It is delivered via intravenous (IV) infusion and is currently under investigation to evaluate its efficacy as a treatment in patients with moderate to severe CD. Although many of the therapies used in the management of UC incidentally produce an anti-angiogenic effect, none have primarily targeted an angiogenic pathway.

1.7 Chronic sequela of Long-term pathological angiogenesis

Colorectal cancer and metastasis

Angiogenesis plays an important role in cancer development and spread. Sustained angiogenesis is one of the six hallmarks of cancer described by Hanahan and Weinberg in 2000 (76). The ability of a tumor to induce and sustain angiogenesis appears to occur in a discrete step via an angiogenic switch. This switch is activated by changing the balance of angiogenic molecules. In some cases there is increased expression of VEGF or FGFs, in others a downregulation of inhibitors such as β-interferon or thrombospondin-1. Patients with UC are at increased risk of colorectal cancer (CRC). The risk depends on disease severity, distribution and duration. Patients with colitis involving the entire large bowel (pancolitis) have a significantly increased risk of developing CRC. A seminal population based study performed in Sweden in 1990

found that patients with pancolitis and a disease duration of >35years had a 30-40% absolute risk of CRC (77). This study led the way for implementation of CRC screening in patients with UC. It is hoped that improved treatment options, surgical technique and CRC surveillance has reduced the risk and allowed early detection of CRC. Indeed more recent studies have shown a lower rate of CRC amongst patients who received biologic therapy (78). Other factors that determine a patient's risk include primary sclerosing cholangitis (PSC) and family history of CRC. Tumor growth and metastasis are angiogenesis dependent. Early studies focus on the effects of angiogenesis as a process important for tumor expansion; however, angiogenesis is critical to tumor metastasis (36, 76). It appears that highly vascular tumors rich in angiogenic molecules metastasis at a higher rate than less vascular lesions. Angiogenesis increases the risk of metastasis by providing a direct route for invasion. Immature and highly permeable tumor vessels allow significant volumes of tumor cells to be shed into the circulation. Although very few of these cells will form tumor metastasis, the number of metastasis usually correlates with the proportional number of cancer cells shed (79). In the case of CRC, metastasis tends to occur through spread to the liver via the portal vein.

Stricture development

Although the inflammatory changes tend to be superficial and mucosal in UC, transmural fibrotic changes and full thickness angiogenesis have been shown to be features of longstanding active UC (80). Scarred deformed colonic mucosa can result

from longstanding disease activity (Picture. 2). Strictures are more common in Crohn's

disease, but if inflammation and pathological angiogenesis is left unchecked, they can

occur in UC. Stricture development can lead to debilitating symptoms and can result in

bowel obstruction and perforation. Stricturing in UC is a significant contributor to

morbidity and is concerning for an underlying neoplasm.
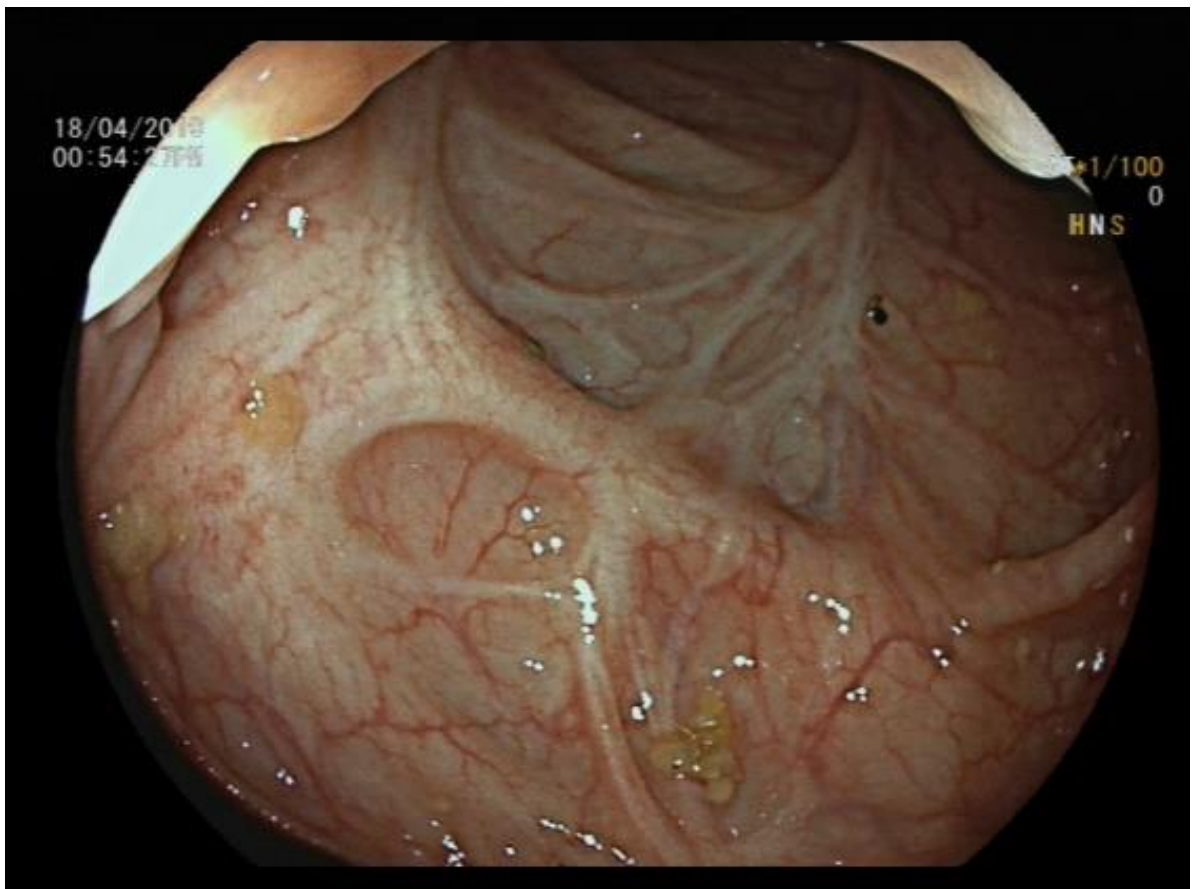
Figure 1.5 Scarred fibrotic changes in the colonic mucosa

Disease severity

Chronic inflammation and pathological angiogenesis are inextricably linked. The long-

term clinical sequelae of these two associated phenomena can be seen in a multitude

of pathologies; psoriasis, rheumatoid arthritis, diabetes mellitus, and cancer. In UC,

the normal vasculature, which is seen endoscopically in healthy colonic mucosa, is obliterated. In assessing a patient with UC at endoscopy, the vascular pattern is often reported and graded as normal, decreased or absent. Absent vascular pattern is associated with increased inflammatory burden and more severe disease. An analysis of the vasculature is incorporated into the Mayo Endoscopic score as well as multiple other disease activity calculators as it gives an indication of disease severity. Pathological angiogenesis forms immature blood vessels and capillaries that are friable and bleed easily. When carrying out an endoscopic assessment on a patient with UC, any contact bleeding (bleeding from contact with the colonoscope) or spontaneous bleeding is noted. Passing blood via the rectum is a major symptom with UC and is caused by disease severity and pathological angiogenesis.

1.8 Anti-angiogenic medications currently licenced

The role of angiogenesis and energy metabolism has been more thoroughly evaluated and therapeutic pathways identified in other medical specialties. Clinically effective anti-angiogenic treatments have been utilised in oncology, rheumatology, dermatology, ophthalmology and haematology.

Bevacizumab, released in 2004, was the first FDA approved anti-angiogenic agent. Its therapeutic effect is elicited through inhibition of VEGF-A. It is administered via intravenous infusion. Neutralising VEGF regresses the vascularisation of neoplasms and inhibits tumour growth by decreasing new vasculature formation. In addition to its

anti-angiogenic effect, it is likely that bevacizumab improves the delivery of chemotherapy by reducing the interstitial pressure in tumors.

Sorafenib is an orally administered multikinase inhibitor that is active against PDGFR, VEGFR and RAF kinases. It is used in oncology to treat hepatocellular (HCC), renal cell and thyroid carcinoma. Sunitinib is another protein kinase inhibitor, it is administered orally. It inhibits multiple receptor tyrosine kinases that are involved in angiogenesis, tumour growth and metastasis. It inhibits PDGFR, VEGFR, stem cell factor receptor (81), colony stimulating factor receptor (CSFR) and Fms-like tyrosine kinase-3. It is licensed for use in gastrointestinal stromal tumors (GIST), renal cell carcinoma and pancreatic neuroendocrine tumors (82).

Ponatinib is a potent oral tyrosine kinase inhibitor. It acts on BCR-ABL, an abnormal tyrosine kinase found in patients with chronic myeloid leukaemia (CML) and acute myeloid leukaemia (AML). The drug can bind mutated and non-mutated forms of the BCR-ABL kinase and as a result confers increased efficacy when compared with similar molecules. Ponatinib also acts against VEGFR, PDGFR and FGFR families of kinases conferring and anti-angiogenic effect.

Tocilizumab is a newly licensed therapy for rheumatoid arthritis (RA). It targets IL-6 blockade and exhibits a potent anti-inflammatory effect. Studies have shown that it inhibits angiogenesis in the synovial tissues of patients with RA in an action that is unique to tocilizumab.

1.9 Complications of targeting angiogenic pathways

Angiogenesis is important in tissue development, growth and healing, indiscriminately blocking angiogenesis entirely is unlikely to be clinically beneficial. Although pathological angiogenesis is likely to perpetuate the chronic inflammatory changes seen in UC it has a role in supplying inflamed tissue with much needed nutrients and oxygen. Patients with UC have micro-circulatory abnormalities that make them more susceptible to ischaemia; profound angiogenic suppression could lead to colonic ischaemia. This has been demonstrated in a number of case reports where potent anti-angiogenic treatments have been given to patients with UC.

Bevacizumab is thought to have caused delayed leakage of sutures in a 50 year old lady with Ulcerative colitis and rectal cancer (83). Similarly, a 62-year-old woman with stage IIIb lung adenocarcinoma developed UC with severe pancolitis following her seventh cycle of Bevacizumab. This was refractory to medical therapy and she went on to have a colectomy (81). Whether bevacizumab induced a flare of UC or caused an ischaemic colitis by virtue of its potent anti-angiogenic effects is unclear. It has been associated with gastrointestinal perforation and fistulae in patients without a background history of IBD (84). Gastrointestinal perforation was first noted in the initial phase 3 clinical trial, 6 cases were recorded (1.5%) in the treatment group vs none in the control arm (85). Similar rates of gastrointestinal perforation have been noted in real world observational data assessing patients receiving this therapy. Bevacizumab has also been linked to gastrointestinal and non-gastrointestinal fistulae formation. Importantly these side effects have not been noted in other agents that

target angiogenic molecules and more recent data has suggested that Bevacizumab is safe to use in patients with IBD (86).

Targeting biological pathways for therapeutic reason inevitably results in unpredictable side effects. Therapeutic manipulation of the VEGF pathway has been associated with an increased incidence of hypertension (87). Clinical safety data suggest that this side effect is dose dependent. It is one of the most frequently observed adverse effects of angiogenic inhibition. In patients receiving sunitinib, the incidence of hypertension was 28% (88). In general, this can be managed with oral anti-hypertensive agents. This side effect is likely caused by vasoconstriction due to reduced nitric oxide synthesis resulting in increased peripheral vascular resistance and hypertension.

There is an increased risk of both venous and arterial thromboembolism in patients receiving potent VEGF inhibitors. Stroke, myocardial infarction and pulmonary embolism can lead to significant morbidity and mortality. Patients with cancer are at increased risk of developing clots; however, evidence suggests that anti angiogenic agents significantly increase their thromboembolic risk. An analysis of 5 randomised controlled trials showed an arterial thromboembolic event rate of 3.8% in the group treated with bevacizumab and chemotherapy, vs 1.7% in those treated with chemotherapy alone ($p<0.01$) (89).

Proteinuria occurs commonly in the setting of VEGF inhibition. The renal glomerulus is made up of podocytes, basement membrane and endothelial cells. VEGF is expressed by podocytes, its inhibition results in proliferation of endothelial cells and loss of endothelial fenestrations resulting in proteinuria. In most cases, it is incidentally found in patients who are asymptomatic and typically decreases after treatment ends. Significant renal injury is rare.

1.10 Study aims

It is our hypothesis that angiogenesis significantly contribute to mucosal inflammation in UC and that our novel patented small molecule drugs will elicit a potent anti-angiogenic and anti-inflammatory effect in human *ex vivo* colonic explant tissue from patient's with ulcerative colitis.

The aims of this translational research thesis are 3-fold:

1) A prospective study to profile the clinical relevance of the expression of angiogenic and inflammatory mediators in UC patients with different degrees of inflammation and response to therapies. The clinical parameters evaluated include patient symptoms, biochemical/stool analysis, mucosal inflammation at endoscopy and histological analysis.

2) Using a human explant model, we will assess the real time release of angiogenic, metabolic and inflammatory mediators from UC colonic biopsies. Examine the action of our 2 novel patented small molecule drugs which target the cysteine leukotriene pathway and inflammation. The outputs of this work will help position the targeting of these processes in the UC setting.

3) Determine how UC colonic explants treated with Quininib drugs can influence the biology of dendritic cells and impact on adaptive immunity.

UC is a chronic and debilitating condition, improved understanding of the colonic microenvironment and the factors that lead to this condition are essential. Pathological angiogenesis has an important role in perpetuating the inflammatory changes seen in UC. Despite this, angiogenesis also plays an important part in wound healing and recovery. Angiogenesis has been studied extensively in other fields and has yielded many new therapeutic pathways have improved the lives of patients. In UC, the study of angiogenesis will provide valuable information on the etiology of this inflammatory condition and could potentially offer therapeutic targets.

Chapter 2: Material and Methods

2.1 Quininib drugs

2.1.1 Parent drug discovery

Work carried out prior to this thesis commencing identified Quininib (Q1), a small molecule drug (<500kDa) which displayed significant anti-angiogenic activity in Tg (flil:EGFP) zebrafish. In previous studies it demonstrated anti-angiogenic effects using *in vitro, in vivo* and *ex vivo* colorectal cancer (CRC) models(90, 91). Q1 was subsequently shown to be as effective as bevacizumab at reducing the secretion of inflammatory and angiogenic molecules from *ex vivo* human CRC tissue. (92).

2.1.2 Quininib analogs

Separate to the thesis structural analogs of quininib were developed to identify novel agents with a more powerful anti-inflammatory and anti-angiogenic effect. These Q1 analogs have been studied in cytotoxic assays, zebrafish and tube formation work. Two lead drugs, pyrazinib (P3) and 1,4-dihydroxy quininib (Q8) were identified. These two patented small molecule drugs now require further examination to advance pre-clinical development. P3 and Q8 were synthesized by Onyx Scientific and Celtic Catalysts. 10mM stock solution was made by dissolving in 100% DMSO and storing at -20°C.

2.1.3 1,4-dihydroxy quininib (Q8)

24 distinct quininib analogs were synthesized and ranked, from this (E)-2-(2-Quinolin-2-yl-vinyl)- -benzene-1,4-diol HCl salt (Q8) emerged as the lead agent, demonstrating robust anti-inflammatory and anti-angiogenic effects. The enhanced potency when

compared with Q1 and other analogs is most likely as a result of the additional hydroxy group phenol ring. The importance of the hydroxy group in other agents such as tamoxifen (licensed anti-oestrogen therapy) have been highlighted by a structure activity relationship study (93).

2.1.4 Pyrazinib (P3)

Pyrazinib (P3), (E)-2-(2-Pyrazin-2-yl-vinyl)-phenol, causes antagonism of CysLT receptor 1. In a separate study, P3 displayed potent anti-metabolic, anti-inflammatory and anti-angiogenic properties when delivered *in-vitro* to isogenic models of OAC and *in-vivo* in zebrafish (94). These effects have been observed with little evidence of toxicity or developmental abnormalities. Importantly P3 demonstrated greater therapeutic activity than its parent drug, Q1. As a result P3 was selected as a lead drug to be further evaluated in other inflammatory and neoplastic model systems.

2.3 Methods

2.3.1 Ethical approval

Ethical approval was granted by the St James's Hospital/Adelaide and Meath Hospital Dublin (SJH/AMNCH) Research Ethics Committee. General Data Protection Regulation (GDPR) came into effect in May 2018. Our procedure for obtaining consent and our consent form/patient information leaflet were amended accordingly. Given the new GDPR and health research regulations (HRR) that also came into effect we submitted

these amendments to the joint research ethics committee and to the Data protection officer (DPO) Trinity college Dublin who both approved the amendments. Patients were given information regarding the study and written informed consent was obtained prior to enrollment. A copy of the information leaflet and consent was given to the patient and another copy left in the medical chart following the procedure. The original consent form was brought to the laboratory and stored in a locked filing cabinet.

2.3.2 Patient Selection

Patients with a formal diagnosis of UC over the age of 17 were included in the study. The diagnosis in each case had been made by a consultant gastroenterologist with special interest in UC. The diagnosis was primarily made based on patient symptoms, characteristic endoscopic appearance and chronicity on histological examination. However, clinical examination, laboratory findings and radiological evaluation were also considered. Patients attending the endoscopy department in St James's Hospital for colonoscopy were offered the opportunity to participate. The patient's basic demographic data was recorded, this included age and gender. Using the electronic patient record (EPR) and medical notes the diagnosis was confirmed, disease duration, distribution and severity were recorded. Patient's medication history was also evaluated with particular focus on medications related to the management of UC. Patients were excluded if they were under 18, were pregnant or unable to give consent.

2.3.3 Assessing ulcerative colitis disease activity

Patient's symptoms were assessed using the Mayo Scoring System for Assessment of Ulcerative Colitis Activity (Table 2.1). The Mayo score is a widely used tool to assess disease activity in patients with UC. Initially described in 1987 by Schroeder et al, it correlates closely with quality of life and is used extensively in research. Scoring is based on stool frequency, rectal bleeding and a global physician assessment. This provides the partial Mayo score. A further score is given at endoscopy to make up the Mayo score. The Mayo score is designed to assess severity of UC in a standardized way and allows response to therapy to be monitored. There is an element of subjectivity with the score as the physician gives their global assessment, and this is given significant weighting. In each case the endoscopist (consultant gastroenterologist, registrar or advanced nurse practitioner) gave the endoscopic sub score. The use of standardized scoring systems reflects a shift in the management of UC, from clinical endpoints (clinical remission/steroid free remission) towards mucosal healing. There is now a focus on treat to target in line with other long-standing conditions (hypertension, COPD). However, evidence also exists showing that the endoscopic sub-score has poor correlation with patient reported symptoms (95).

Table 2.1 *Mayo score for assessing activity in Ulcerative colitis*

This table outlines the parameters assessed for the Mayo score. Scores are interpreted: 0 to 2: Remission, 3 to 5: Mild, 6 to 10: Moderate, > 10: Severe disease activity.

Parameter	Description	Score
Stool frequency	Normal	0
	1 to 2 stools/day more than normal	1
	3 to 4 stools/day more than normal	2
	>4 stools/day more than normal	3
Rectal Bleeding	None	0
	Blood visible <half of the time	1
	Blood visible half of the time or more	2
	Passing blood alone	3
Mucosal appearance at endoscopy	Normal	0
	Mild (erythema, decreased vascular markings)	1
	Moderate (Marked erythema, erosions)	2
	Severe (Bleeding, ulcers)	3
Physician Assessment	Normal	0
	Mild	1
	Moderate	2
	Severe	3

2.3.4 Colonoscopic assessment of ulcerative colitis

The colonoscopy was performed by either a specialist registrar, advanced nurse practitioner or consultant gastroenterologist. Two endoscopy nurses were present during the procedure. Patients were positioned in the left lateral position. All patients had an intravenous (IV) cannula inserted into their arm to deliver conscious sedation. IV midazolam and fentanyl were given prior to starting the procedure. Vital observations were checked prior to and after sedation was given as well as throughout the procedure. A high definition colonoscope was used for all procedures (Fujifilm Eluxeo 700 series Colonoscopes). The colonic mucosa was carefully examined and scored in accordance with the Mayo endoscopic score. Note was taken of the vascular pattern, the presence of ulcers, bleeding, polyps and the distribution of disease. In some cases, multi-light illumination technology in the form of linked color imaging

(LCI) and blue light imaging (BLI) was deployed to further characterize colonic mucosa or polyps. Photographic evidence of caecal/Terminal ileal intubation was recorded. In accordance with best practice the colonoscope was withdrawn over greater than 6 minutes. Any concerning polyps were resected, pseudo polyps in general were not removed, but were sampled if there were any atypical features. 4 biopsies from inflamed and/or 4 biopsies from non-inflamed colonic mucosa were taken were taken for research purposes. The samples were placed in saline soaked gauze in a sterile container and brought to the laboratory to be cultured immediately. As per usual practice, routine biopsies were also taken, placed in formalin and brought to histopathology. The endoscopist rated the quality of the bowel preparation and scored the patients comfort level following the procedure. Any immediate complications noted during the procedure were recorded.

2.3.4 Laboratory results

Patients attending for colonoscopy had laboratory results recorded. Laboratory results relevant to UC and markers of inflammation were documented and included C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), white cell count (WCC), albumin and haemoglobin (Hb).

C-reactive protein (CRP)

CRP is a marker of acute inflammation released by hepatocytes in the liver in response to circulating interleukin-1β, interleukin-6 and TNF-α (96). It is measured in milligram per litre (mg/L), with a normal reading <5mg/L. CRP rises rapidly in response to an

inflammatory stimulus with increased number of hepatocytes recruited. CRP levels drop quickly following resolution of inflammatory stimulus with a half-life of 48 hours. CRP has been shown to correlate with clinical and endoscopic UC activity (97).

Erythrocyte sedimentation rate (ESR)

ESR is a measure of how quickly erythrocytes from a blood sample settled at the bottom of a test tube. This normally occurs in a slow predictable manner, but in states of inflammation will occur more quickly. ESR is measured in milimetres per hour (mm/hr) the normal range may vary between laboratories but is often 0 to 22mm/hr, with findings above that suggestive of inflammation. In a paediatric IBD population the role of ESR and CRP was evaluated, they found both markers can accurately reflect disease activity, with CRP slightly more effective (98).

Albumin

Albumin is a protein synthesized by the liver, it acts as a substrate on which hormones and fatty acids are transported. Albumin is key in maintaining oncotic pressure, ensuring extracellular fluid volumes are stable. It is measured in gram per litre (g/L) with a normal range of 35-55g/L. Albumin is important in assessment of ulcerative colitis as it is an inverse marker of inflammation. A low serum albumin is associated with increased inflammatory burden and more severe disease activity. In active UC there is increased enteric protein loss which further strengthens the use of albumin as a marker of disease severity (99). Albumin also plays an important role in the

pharmacokinetics of drug delivery in UC. Serum albumin level was a predictive factor

in clinical response amongst UC patients receiving infliximab (100).

Haemoglobin (Hb)

Hb is the red iron containing protein that transports oxygen in the blood from the

lungs to the rest of the body. It is measured in gram per decilitre (g/dL), with a normal

range 13.5 to 17.5g/dL in men and 12 to 15.5g/dL in women. A reduced Hb (anaemia)

in UC is suggestive of increased disease activity. Acute blood loss through rectal

bleeding leading to Hb drop is indicative of severe disease. Given the inflammatory

nature of UC a chronic depletion of iron stores may also result in anaemia (101).

2.3.5 Histopathological assessment of UC colonic biopsies

All samples were analysed by two histopathologists with special interest in GI

pathology. Firstly, colonic biopsies were described as inflamed or non-inflamed. The

level of inflammation was then graded as mild, moderate or severe. The grading was

decided by the histopathologists based on architectural changes, basal plasmacytosis,

crypt destruction, erosions and ulceration.

2.3.6 Generation of tissue cultured media (TCM)

The samples were immediately brought to the lab for processing. 24 well plates were

used, in one plate (wash plate) 4 wells for each sample were filled with wash buffer

(PBS-Sterile, 10% FCS, 1% Penicilin/Streptomycin, 0.1% Gentamicin, 1% Fungizone). In

another plate (culture plate) one well for each sample was filled with 900 ul of media

(M199, 10% FCS, Penicillin/Streptomycin, 0.1% Gentamicin, Fungizone, Insulin) and

drug/control (100ul). Each biopsy was washed 4 times in different wells then

transferred into the culture plate which was wrapped in parafilm to stop evaporation

during the incubation period. The plate was placed in a CO2 (5%) incubator at 37°C for

24 hours. After 24 hours the biopsies and TCM were removed in the fume hood. The

biopsies were snap frozen with liquid nitrogen and both the biopsies and TCM were

stored in a freezer at -80°C.

2.3.7 Protein extraction

One metal bead and 200µL RIPA+ were added to each tube. Tubes were brought to

the TissueLyser II (Qiagen) and placed in the trays, ensuring both trays were balanced.

The trays were placed in the machine, wheels tightened, and lid closed. The

TissueLyser was set to 25 Hz for 2 minutes. The samples were transferred to clean cold

Eppendorf tubes. The fluid was removed with yellow pipette tip, then the bead was

poured into the lid and removed. Any residual fluid was removed thereafter. The

samples were spun for 20mins at 13,000rpm at 4°C. Then transferred to a new tube.

2.3.8 Protein quantification

Pierce BCA protein assay kit by ThermoFisher was used to quantify total protein from

explant sample in microgram per millilitre (µg/ml). The kit formulation based on

bicinchoninic acid (BCA) combines the reduction of Cu^{+2} to Cu^{+1} (the biuret reaction) in

an alkaline medium with very accurate detection of Cu^{+1}. Chelation of BCA with one

cuprous ion gives a purple coloured reaction. The water-soluble component displays

strong absorbance at 562nm that is linear with increasing protein content over a broad

range (20-2000 µg/ml). Although the final colour continues to develop, this rate is

sufficiently slow to permit large numbers of samples to be processed together. Two

assay procedures are described for this kit, the microplate procedure was chosen as it

requires a smaller volume of sample. The contents of one albumin standard were

diluted into vials, using the same diluent as the explants. Table 2.2 was used as a guide

to make a set of protein standards.

Table 2.2 *Preparation of diluted albumin (BSA) standards, from ThermoFisher.*

Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working Range = 20–2,000 µg/mL)			
Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

2.3.9 Preparation of the working reagent

The following formula was used to calculate the volume of working reagent needed:

(Number of standards and unknowns) x (Number of replicates) x (volume of working

reagent per sample) = Volume of working reagent required.

Working reagent was prepared by adding (50:1) Reagent A:B.

2.3.10 Microplate procedure

25µL of each unknown and standard sample replicate were pipetted into 96-well

microplate. 200µL of working reagent was added and mixed thoroughly for 30 seconds

on a plate shaker. The plate was covered and incubated for 30mins at 37°C. Then

allowed to cool to room temperature and the absorbance on the plate reader at

562nm was read. The average absorbance reading of the blank standard replicates

were subtracted from the readings of all the other individual standards and unknown

replicates. A standard curve was prepared with the average blank corrected

measurement for each BSA standard vs its concentration in µg/mL. From this the

protein concentration of each unknown was calculated using the standard curve.

2.3.11 Ulcerative colitis explant ELISAs

The angiogenic and inflammatory microenvironment of the UC explants were

characterised by carrying out ELISAs on the TCM generated following explant culture.

Meso-Scale Discovery (MSD) V-Plex proinflammatory and angiogenic human panel 1

kits were used. The proinflammatory panel contains the following analytes: TNF-α,

IFN- γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13. These 10 cytokines are

important in inflammatory response and immune system regulation along with many

other biological processes. The angiogenic panel measures 7 proangiogenic

biomarkers: FGF (basic), VEGF-A, VEGF-C, VEGF-D, VEGFR-1/Flt-1, PIGF, Tie-2. Both

panels are sandwich immunoassays, the plate is precoated with capture antibodies on

separate well-defined spots. MSD buffer is added to create the appropriate

environment for electrochemiluminescence (ECL), the plate reader applies a voltage to

the electrodes within the plate causing the captured labels to emit light. V-PLEX assay

kits have been validated in accordance to the principles in "Fit for Purpose method

development" (102).

2.3.12 V-Plex angiogenesis ELISA

Reagent preparation

Diluents and buffers were brought to room temperature. The stock calibrator was thawed on wet ice for at least 30 minutes to prepare calibrator solutions. To prepare the highest calibrator, 20 µL of stock calibrator was added to 380µL of diluent 7 and mixed by vortexing. Serial dilution was repeated 5 times to generate 7 calibrators. Diluent 7 was used as the Zero calibrator. Samples and controls were diluted 2-fold in diluent 7 before adding to the plate. Combined detection antibody solution was prepared by diluting each detection antibody 50-fold in diluent 11.
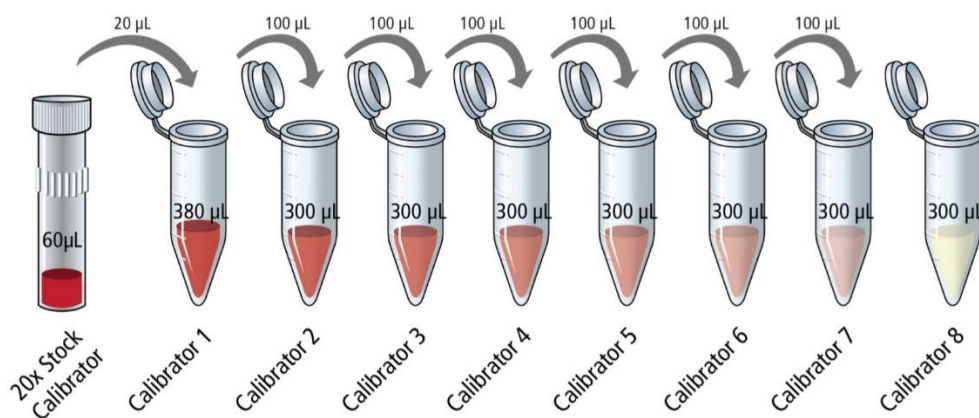
Figure 2.1 *Dilution schema for preparation of calibrator standards.*

Assay Protocol

150µL of blocker A solution was added to all wells and sealed with adhesive plate seal. It was incubated at room temperature with shaking for 1 hour. Then washed 3 times with at least 150µL/well of wash buffer. 50µL of diluted sample, calibrator or control

was added per well. The plate was sealed and incubated at room temperature with shaking for 2 hours. Again, the plate was washed 3 times and 25µL of detection antibody solution was added to each well. The plate was sealed and incubated at room temperature with shaking for 2 hours. Finally, the plate was washed a third time, 150µL of read buffer T was added to each well. The plate was then analysed on the plate reader.

2.3.13 V-Plex Pro-inflammatory ELISA

Reagent Preparation

All reagents were brought to room temperature. The multi-analyte calibrator was reconstituted in 1,000µL of diluent 2 (the highest calibrator). After reconstituting it was inverted 3 times. This was left to equilibrate at room temperature for 30 minutes and then vortexed using short pulses. The next calibrator was prepared by transferring 100µL of the highest calibrator to 300µL of diluent 2. This was mixed well by vortexing and the previous step was repeated 4-fold to generate 7 calibrators. Diluent 2 was used as zero calibrator.
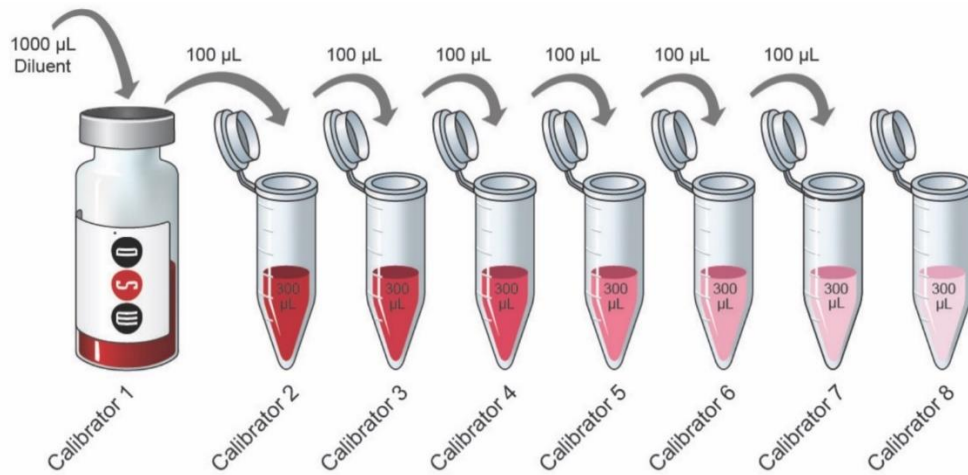
Figure 2.2 *Dilution schema for preparation of calibrator standards.*

Assay protocol

The plate was washed 3 times with 150μL/well of buffer wash. 50μL of prepared

samples, controls or calibrators were added per well. The plate was sealed with

adhesive plate seal and incubated at room temperature with shaking for 2 hours. The

plate was washed again and 25μL of detection antibody solution was added. The plate

was sealed and incubated at room temperature with shaking for 2 hours. Finally, the

plate was washed a third time, 150μL of 2X Buffer T was added to each well. The plate

was then analysed.

2.4 Protocol for human Dendritic cell (DC) generation from peripheral blood mononuclear cells (PBMC)

2.4.1 Peripheral Blood Mononuclear Cell (PBMC) isolation

Blood packs were collected from St James's Hospital Blood Transfusion Unit. Packs were sprayed with 70% ethanol and the tube cut with sterile scissors in laminar flow. EDTA (1 ml of 0.5 mM, sterile from Sigma) was added to buffy coat. The blood was transferred into 50ml tubes and diluted 1:3-1:4 (at least) with sterile phosphate-buffered saline (PBS). 35ml of diluted blood was carefully layered over 15ml Ficoll-Hypaque/Lymphoprep in a 50ml tube. The blood was centrifuged at 2,000 revolution per minute (RPM) for 20minutes with the brake off. A pipette was used to remove the yellow liquid to the level of the buffy coat interface, this was discarded into a bucket of bleach. The white PBMC layer was carefully removed and transferred into a 50ml tube containing Roswell Park Memorial Institiute Medium (RPMI) and sterile PBS. This was centrifuged at 1,800 rpm for 10 minutes. The supernatant was discarded and the pellet resuspended in RPMI. This was centrifuged at 1,200 rpm for 10 minutes. A further wash was carried out for 7 minutes at 1,100 rpm to remove platelets. Cells were counted using trypan blue in a haemocytometer.

2.4.2 Monocyte isolation

The MACS magnetic bead isolation kit was used for CD14 selection. Cells were washed in MACS buffer and resuspended in 2.7ml (80μl per 107 cells). 300I CD14 beads were added and incubated in the fridge for 15 minutes at 4°C. Another wash was carried out

in 30-50ml MACS buffer and centrifuged at 4°C for 10 minutes. Again resuspended in 1-2ml MACS buffer. The LS column washed again with 3ml MACS buffer. The sample was applied to the LS column and negative fraction collected (purity testing). A final wash was performed with 3ml MACS buffer, the column was removed from the magnet and CD14+ cells eluted in 5mls. CD14 cell purity check was carried out. An aliquot of PBMC was stained, positive fraction and negative fraction with CD14-FITC (2μl per 50μl cells), purity was assessed by flow cytometry. Following culture for 7 days purity of immature DCs was checked, with expectation that DCs would be CD14 negative, CD209 positive and CD11c positive. Macrophages were expected to be CD14 positive and CD209 negative.

2.4.3 Dendritic cell generation from monocytes

Monocytes were counted and resuspended at 1×10^6 cells per ml in RPMI. All cells obtained were plated as only approximately 30% will become DCs. Granulocyte-macrophage colony-stimulating factor (GM-CSF) 50ng/ml and 70ng/ml IL-4 was added. 3ml of cell suspension was plated per well in a 6 well plate and placed in an incubator for 7 days to allow differentiation of monocyte precursors into immature DCs. After 3 days, 1.5ml of cell culture medium was gently replaced with fresh medium containing 100ng/ml GM-CSF and 140ng/ml IL-4. Great care was taken to disturb plates as little as possible, vortexing was avoided and gentle pipetting was used instead to resuspend DC suspension. A maturity test was carried out on day 5, a small amount of immature DCs were plated in LPS positive and negative settings for 24 hours and flow cytometry performed to check maturity status.

2.4.4 Explant TCM Dendritic cell work

Explant TCM was plated in 96 well round bottom plates. One LPS positive and one LPS negative plate. Media only controls (RPMI) were plated X10. M199 only controls were plated X10. Drug controls were also plated. Immature DCs were harvested on day 6 by careful pipetting and washing of wells with sterile PBS. DCs were counted and plated out in 96 well round bottom plates at 0.2×10^6 cells in 200μL for 5 hours. Then the desired maturation stimulus was added to the LPS positive induced plate only for 16hours. Not all monocytes differentiate into DCs, many remain stuck to wells despite multiple washes.

2.4.5 Dendritic cell flow cytometry

Mastermix antibody was prepared as per Tables 2.2-2.3. Prior to reconstitution the vial of Zombie was spun down in a microcentrifuge to ensure the reagent was at the bottom of the vial. The kit was preheated to room temperature, 100μl of DMSO was added to one vial of Zombie UV dye and mixed thoroughly until fully dissolved. Zombie UV dye was diluted at 1:10 in PBS. 1μl of Zombie UV dye was added for each well to the Mastermix antibody. Mastermix antibody was diluted in PBS buffer and kept in the dark at 4°C. Cells were spun and supernatants removed, supernatants were kept for IL-10 an dIL-12p70 ELISA. Cells were washed in PBS buffer in the 96 well plates X2. Cells were then incubated at room temperature, in the dark for 30 minutes with 100μl of Mastermix. After this, cells were washed with 100μl of FACS buffer and fixed with 100μl of 1% paraformaldehyde for 30 minutes at 4°C. A final wash was carried out with 100μl PBS, cells were centrifuged and resuspended in 200μl of FACS per well.

Table 2.3 *Immaturity test plated on Day 5 → Flow on Day 6*

Immaturity test	BD/Biolegend Antibodies	Facs Canto
Marker	Fluorochrome	10 X (ul)
Lineage	FITC	5
CD80	PE	3.5
CD83	PeCy7	3.5
CD86	PerCpCy5.5	3.5
CD11c	BV510	2.5
HLA-DR	APC Cy7	1
Master mix volume		19 ul
Add FACs Buffer		981 ul
Final Volume		1000 ul

Table 2.4 *Dendritic Maturation analysis plated on Day 6 → Flow on Day 7*

DC Maturation Marker	BD/Biolegend Antibodies	Facs Canto		
Marker	Fluorochrome	10 X (ul)	100 X (96wd)	200X (96wd)
CD80	PE	3.5	35	
CD83	PE-Cy7	3.5	35	
CD86	PerCp-Cy5.5	3.5	35	
CD11c	BV510	2.5	25	
CD40	FITC	4.5	45	
CD54	APC	1	10	
HLA-DR	APC Cy7	1	10	
PD-L1	BV421	7	70	
Master mix volume		26.5 ul	0.265 ml	0.53 ml
Add FACs Buffer		473.5 ul	4.735 ml	9.47 ml
Final Volume		500 ul	5 ml	10 ml

2.5 Statistics

Data from electronic patient records (EPR) was pseudo-anonymised and recorded in an excel document. Basic demographic data was analysed using excel and SPSS statistics version 26 from IBM. UC clinical characteristics were evaluated with SPSS. Clinical correlations were calculated on R-studio with data graphed using the corrplot package (R-studio). Pearson's correlation was used to compare continuous variables and spearman's rank order test was used to evaluate to relationship between ordinal variables. A correlation was considered strong if it was >0.7, moderate $0.4-0.7$ or weak <0.4. MSD multiplex Elisa was analysed with SPSS, Wilcoxon signed rank tests were carried out to analyse correlation between drug and control analytes. GraphPad prism 8 was used to graph analyte correlations. P values of <0.05 were taken to be significant with P values <0.001 very significant.

Chapter 3:

Profiling the clinical relevance of the expression of angiogenic and inflammatory molecules in ulcerative colitis with varying degrees of severity

3.1 Introduction

In this chapter the UC colonic microenvironment will be characterised by profiling the secretion of angiogenic and inflammatory molecules. The significance of these molecules will be revealed by correlating molecule expression with the clinical data.

There is a constant drive to better understand the nature of UC. Whether increased knowledge of etiology, pathogenesis or therapeutics, the drive to fully understand and treat UC has been important ever since it was first diagnosed by Wilks and Moxon in 1875 (103). As with many other medical conditions a physician can assess disease severity by accurate history taking and thorough examination. Scoring systems such as the Mayo score are commonly used to quantify disease activity. The accuracy with which the Mayo score predicts disease activity and the correlation with analyte release in the colonic microenvironment will be evaluated in this chapter.

The advent of laboratory testing and radiological examination afforded further important clinical data that can influence treatment options. C-reactive protein (CRP) is a sensitive marker of systemic inflammation and has been shown to predict severe UC flares (97). In this chapter, the relationship between serum CRP and the molecules secreted in the UC colonic microenvironment is studied. In gastroenterology, the physician is given the almost unique opportunity to directly witness the disease process through endoscopic assessment. Performing colonoscopy also allows sampling to be carried out, histopathological assessment as well as stool analysis can give further important clinical information. Although colonoscopic assessment is the gold

standard for diagnosis there are significant risks associated with colonoscopy including colonic perforation or bleeding (104). In this chapter the characteristics reported at colonoscopy will be evaluated and the relationship with inflammatory and angiogenic analyte levels assessed. Many non-invasive means of assessing disease severity have been developed, the most commonly used being faecal calprotectin testing which is an accurate marker of colonic inflammation (105). This stool test is very useful in assessing disease activity and response to therapy. In many cases avoiding the need for colonoscopy. Despite the wealth of clinical data available our understanding of ulcerative colitis is still lacking.

3.2 Aims

The aim of this chapter is to profile the clinical relevance of angiogenic and inflammatory molecules in patients with ulcerative colitis. Clinical information including patient's symptoms, laboratory findings, colonoscopic and histological data will be thoroughly evaluated. The relationship between these clinical parameters and the inflammatory and angiogenic colonic microenvironment will be studied.

The aims in this chapter are:

1. Profile the clinical characteristics of a UC patient cohort.

2. Evaluate for correlations between clinical scores and laboratory results.

3. Evaluate for crosstalk between angiogenic and inflammatory analytes within the colonic microenvironment.

4. Study the relationship between clinical scores and analyte expression.

5. Evaluate the effect of biologic exposure on analyte secretion in the colonic microenvironment.

3.3 Patient population and disease characteristics

26 patients with ulcerative colitis participated in the study. 13 male and 13 female patients (Table 3.1). The mean age was 54 years, ranging from 21 to 79 years of age. The colonic disease distribution was recorded; 9 patients had pancolitis involving all the large bowel, 11 had left-sided colitis and 6 had proctitis involving only the rectum. The mean duration of disease was 12 years (range 1-61 years). 46% of patients had a diagnosis of UC for greater that 10 years. The partial Mayo score based on stool frequency, rectal bleeding and global assessment was calculated and used as a marker of patient's symptoms. The mean score was 3 (range 0-7). However, 42% of patients has a partial Mayo of 0. The Mayo endoscopic score was given following colonoscopy and recorded in the procedure report. This score rated colonic inflammation from 0 to 3 depending on the level of severity. The mean score was 2 (range 0-3). 20% of patients were given an endoscopic Mayo score of 3 suggesting severe colitis. 23% were scored as a 2, equating to moderate colitis. 54% were scored as a 1, mild colitis. With one patient given an endoscopic Mayo score of 0, remission. Both the partial and endoscopic Mayo scores were added to give the total Mayo score. The total Mayo score is a well recognised scoring system for assessing UC disease activity. The mean total Mayo score was 4, with a range of 0-10. 38% of patients had a score from 6-10, moderate disease activity. 42% were scored 0-2, indicating remission. The remaining 20% had mild disease activity (score of 3 to 5). Other data recorded from the colonoscopy included; appearance of the vascular pattern, presence of ulcers,

pseudopolyps or bleeding. 54% of patients had a colonic vascular pattern that was reduced or absent when described by the endoscopist. 23% of patients had evidence of colonic ulceration and only 12% had active bleeding at the time of colonoscopy. Pseudopolyps were reported in 35% of cases.

Routine colonic biopsies were taken along with samples for research in all procedures. These biopsies were evaluated by a consultant histopathologist with special interest in IBD. 58% of patients had inflammatory changes consistent with active UC, however in only 8% of cases this was classified as severe. In two patients dysplasia was found, in both cases this area of dysplasia was within an identified polyp and did not involve a segment of bowel where active colitis was present. In both these patients the dysplasia was low grade and the polyps were removed with a satisfactory margin. 5 patients had required hospital admission for management of a UC flare within the last 12 months. To date, one patient has gone on to require colectomy.

Table 3.1 *Patient demographics and clinical characteristics.*

Gender		No. of Patients
	Male (%)	13 (50)
	Female (%)	13 (50)
Age (yr), mean (Range)		54 (21-79)
UC Distribution		
	Pancolitis (%)	9 (35)
	Left Sided Colitis (%)	11 (42)
	Proctitis (%)	6 (23)
Disease Duration (yr), mean (range)		12 (1-61)
MAYO Score (range)		
	Total, mean	4 (0-10)
Pseudopolyps		
	Yes (%)	9 (35)
	No (%)	17 (65)
Bleeding		
	Yes (%)	3 (12)
	No (%)	23 (88)
Inflammation on Histology		
	Yes (%)	15 (58)
	No (%)	11 (42)
Previous Dysplasia		
	Yes (%)	2 (8)
	No (%)	24 (92)
Medications		
	5-aminosalicylic acid (%)	17 (71)
	Azathiprine (%)	6 (23)
	Current Biologic (%)	9 (35)
	Biologic Type:	
	Infliximab (%)	2 (8)
	Adalimumab (%)	1 (4)
	Golimumab (%)	2 (8)
	Ustekinumab (%)	1 (4)
	Vedolizumab (%)	3 (12)
	>/=2 Biologics (%)	6 (23)
	Current Steroids (%)	18 (69)
Hospital Admission in the last year		
	Yes (%)	5 (19)
	No (%)	21 (81)

3.4 Biologic therapies in UC patient cohort

9 patients were receiving biologic therapy at the time of colonoscopy. Various biologic

agents were being prescribed including infliximab, adalimumab, vedolizumab,

golimumab and ustekinumab (Figure 3.1). All patients in the biologic exposed group

had received anti-TNF therapy at some stage, patients being administered

vedolizumab and ustekinumab were previously on infliximab (Anti-TNF). 42% of

biologic exposed patients were on steroids therapy at the time of colonoscopy and

70% had received at least one course of steroids in the last year. 65% of patients were

receiving 5-aminosalicylic acids (5-ASA) either orally or topically in the form of a

suppository or enema. 23% of patients were taking azathioprine. A range of other

(non-UC) medications were being taken by patients. When crosschecked with a drug

interactions database no significant interactions were found. The most common

medications included aspirin (3 patients), lansoprazole (3 patients), metformin (2

patients) and atorvastatin (2 patients). However, the majority of patients (62%) were

not receiving any additional medications excluding their UC treatment.
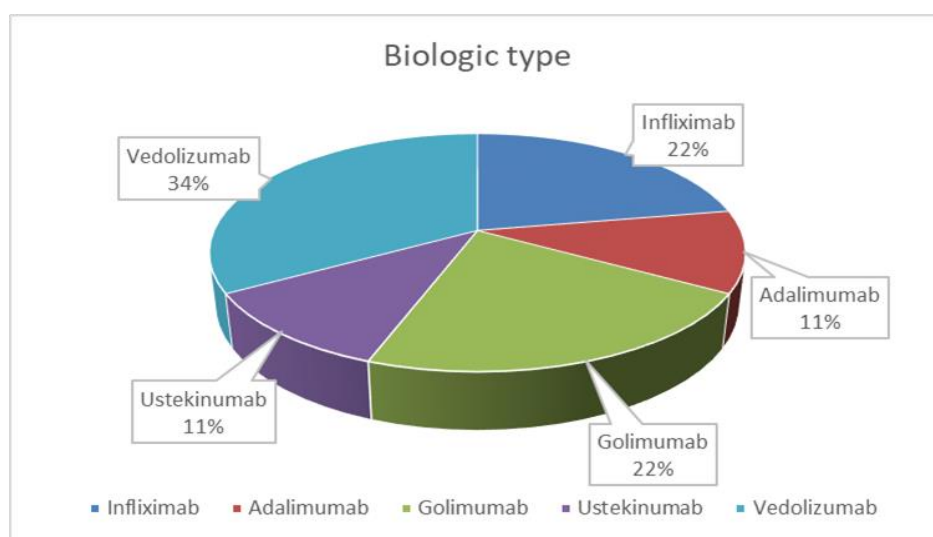
Figure 3.1 *Biologic exposed patient cohort.*
Biologic type in the patient cohort receiving therapy at the time of colonoscopy (n=9).

3.5 Relationship between clinical parameters

Correlations were performed to evaluate the relationship between symptom scores, biochemical abnormalities, and disease severity at endoscopy. These were plotted with corrplot from R studio (Figure 3.2). A correlation was considered strong if >0.7, moderate if 0.4-0.7 and weak if <0.4. The partial Mayo score correlated significantly with the total Mayo score (r=0.978, P value=0.000) and endoscopic sub score (r=0.607, P value= 0.001). A higher partial Mayo score was also associated with reduced or absent vascular markings and bleeding. The partial Mayo score also showed moderate correlation with the presence of inflammation at histological examination (r= 0.435, P Value 0.026). Increasing degrees of inflammation (mild/moderate/severe) were associated with a higher symptom score (r=0.479, P value= 0.013). However, the endoscopic Mayo score displayed a more significant correlation with the presence of histological inflammation (r=0.588 ,P Value=0.002) and the grade of inflammation (r=0.737, P Value=0.00).

CRP displayed a strong positive correlation with bleeding at endoscopy (r=0.859, P value=0.000). CRP correlated with total Mayo score (r=0.526, P value=0.006) and the Mayo sub-scores. CRP was also significantly associated with reduced vascular pattern (r=0.451, P value=0.021), presence of colonic ulcers (r=0.574, P value=0.002) and hospitalisation within the last year (r=0.534. P value= 0.005). CRP and Albumin had a significant inverse relationship (r=-0.585, P value=0.002). Faecal calprotectin was a poor marker of disease activity, although it did display a non-significant moderate positive correlation with Mayo endoscopic sub-score (r=0.405, P value=0.069).

Patients with increased disease duration were less likely to be receiving steroid therapy (r= -0.393, P value= 0.047). The disease distribution and the presence of pseudo-polyps did not correlate with any of the clinical scores. Biologic therapy was moderately associated with hospital admission in the last year (r=0.659, P Value=0.00). Although biologic therapy did not correlate significantly with any of the scoring systems an association with ulcers at endoscopy and the grade of inflammation at histology was noted. Steroid and 5-ASA therapy were inversely associated with disease duration although this did not reach statistical significance. Steroid therapy displayed a mild/moderate positive correlation with partial Mayo score (r=0.399, P value=0.044), total Mayo score (r= 0.415, P value= 0.035) and faecal calprotectin (r=0.458, P value=0.037).
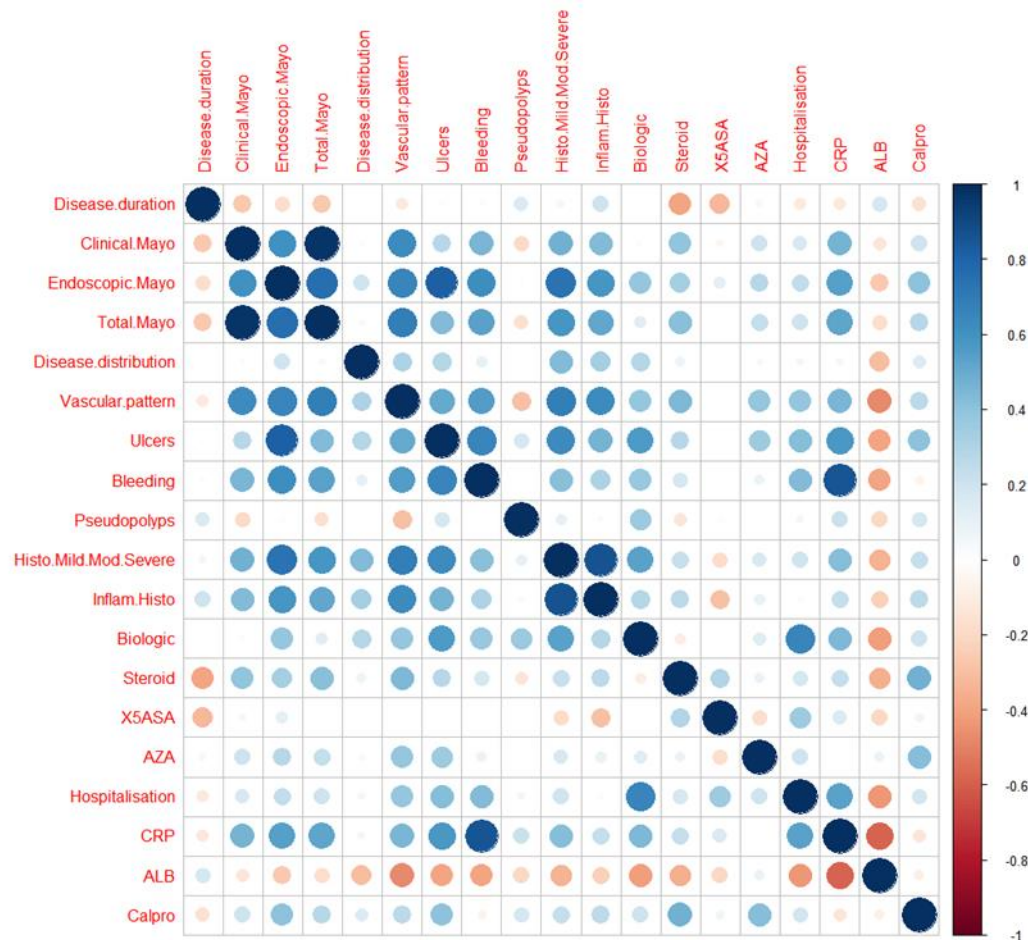
Figure 3.2 *Corrplot of clinical parameters* .
Clinical Mayo score correlates significantly with other Mayo scores and reduced vascular pattern. CRP correlates significantly with bleeding at endoscopy, all Mayo scores, and displays inverse relationship with albumin.

3.6 C-reactive protein (CRP) as marker of disease severity

The role of CRP as a marker of disease severity was further analysed by dividing the patients into high (>5mg/L) and low CRP (<5mg/L) groups. Mann Whitney U tests were performed to compare clinical parameters in both groups (Figure 3.3). The patient group with an elevated CRP had increased disease severity at endoscopy (P value= 0.0069). A high CRP was also associated with higher clinical (P value= 0.0275) and total Mayo scores (P value= 0.0212). An elevated CRP was also significantly associated with

increased erythrocyte sedimentation rate (P value=0.0018). Haemoglobin (Hb) levels in

the high CRP group were lower, but this did not reach clinical significance.
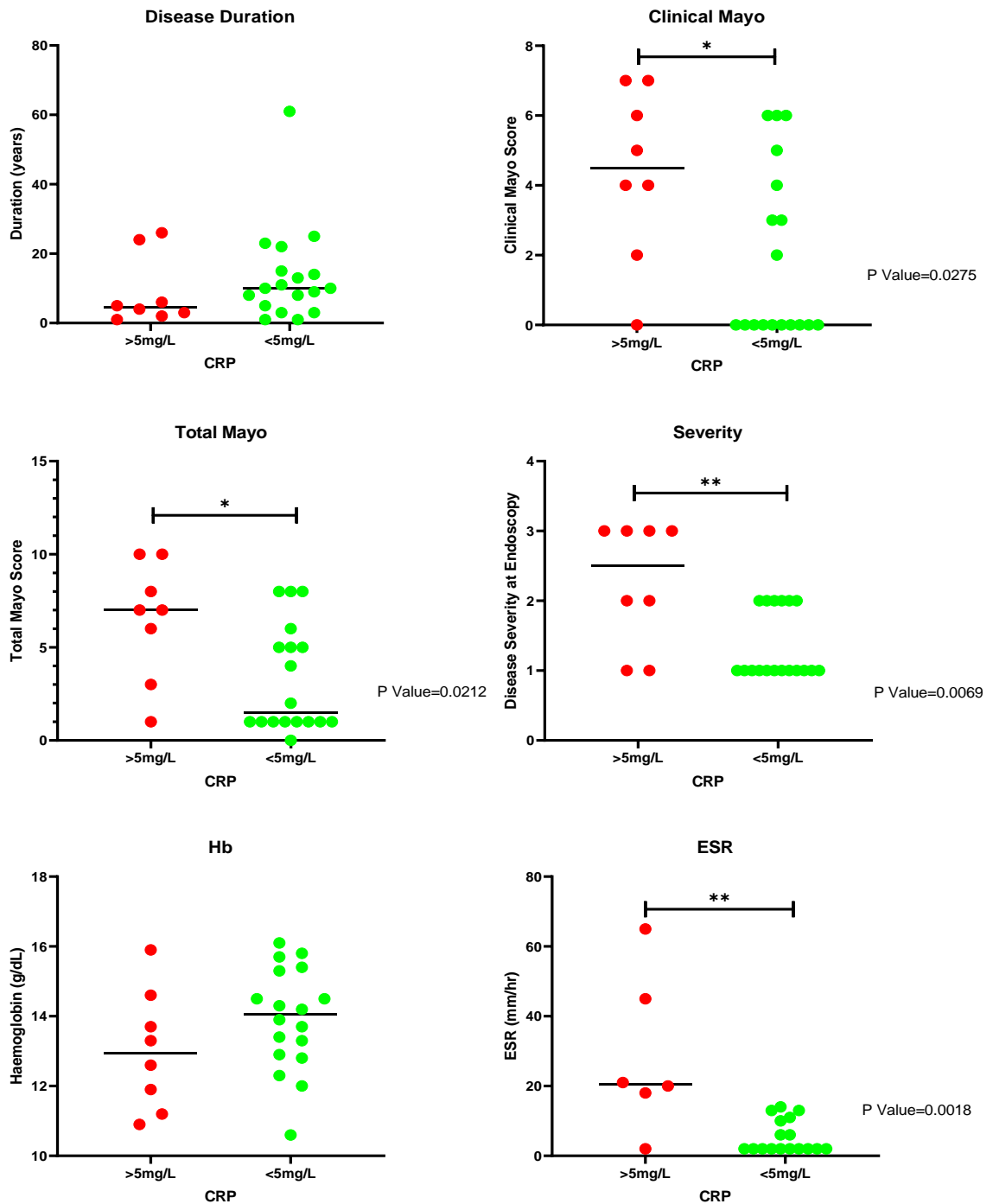
Figure 3.3 *Clinical parameters in high (>5g/dL) and low (<5g/dL) CRP groups.*
Higher CRP was associated with increased Mayo scores, disease severity at endoscopy
and erythrocyte sedimentation rate (ESR). Mann Whitney U Test. Line denotes
median.

3.7 Inflammatory and angiogenic analyte secretion

Multiplex meso scale discovery (MSD) ELISAs were performed on the UC colonic explants. An inflammatory panel was performed to evaluate secretion of interferon-γ (INF- γ), tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-12p70 (IL-12p70) and interleukin-13 (IL-13). An angiogenic panel was performed to calculate secretion of basic fibroblast growth factor (bFGF), vascular endothelial growth factor receptor 1 (Flt-1), placental growth factor (PIGF), angiopoietin receptor (Tie-2) and vascular endothelial growth factor (VEGF) A, C and D. Figure 3.2 displays the readings for each analyte, all readings are normalized to protein content.
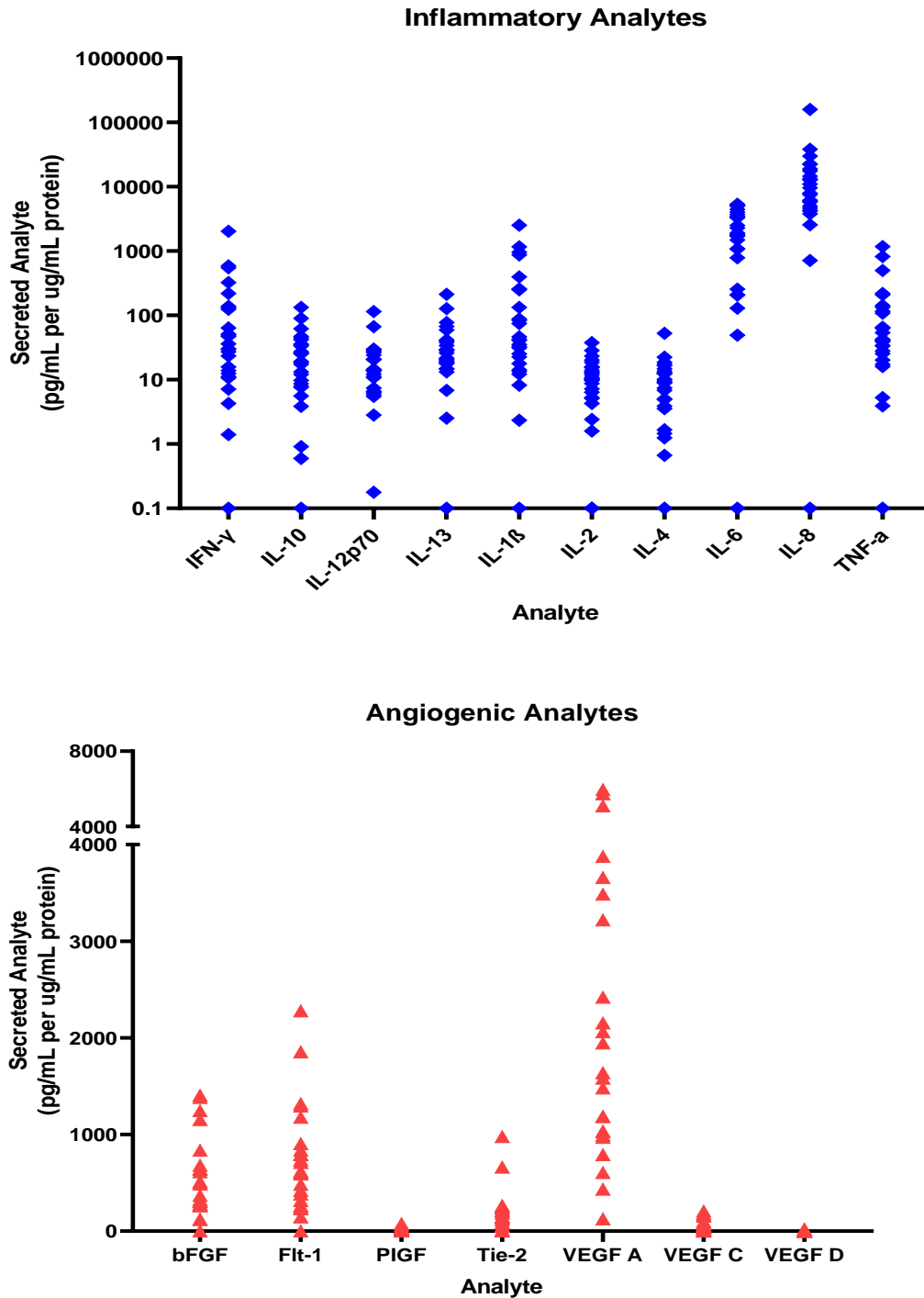
Figure 3.4 *Secreted inflammatory and angiogenic analytes from UC explants.*
Angiogenic and inflammatory analyte readings from n=26 ulcerative colitis colonic biopsies treated with DMSO for 24hours and evaluated by multiplex ELISA. Data normalized to protein content. (Above) Secreted levels of inflammatory molecules Interleukin(IL)-1β, 2, 4, 6, 8, 10, 12p70, 13, tumor necrosis factor(TNF)-α, interferon(IFN)-γ from colonic explants. (Below) Secreted levels of angiogenic proteins basic fibroblast growth factor (bFGF), vascular endothelial growth factor receptor 1 (Flt-1), placental growth factor (PIGF), angiopoietin receptor (Tie-2) and vascular endothelial growth factor (VEGF) A, C and D.

3.8 Correlations between inflammatory and angiogenic analyte expression

Significant crosstalk was noted between the angiogenic and inflammatory analytes in the UC colonic microenvironment (Figure 3.5). Inflammatory cytokine TNF-α exhibited a positive correlation with angiogenic analytes Flt-1 (r=0.611, P value=0.001) and bFGF (r=0.471, P value=0.15). It was also associated with increased levels of inflammatory analytes IL-1β (r=0.865, P value=0.000) and IL-6 (r=0.508, P value=0.008). Angiogenic molecule VEGF A correlated positively with inflammatory analytes IL-2 (r=0.618, P value=0.001), IL-6 (r=0.401, P value=0.042), IL-10 (r=0.655, P value=0.000) and IL-13 (r=0.538, P value=0.005). Similarly PIGF correlated significantly with multiple interleukins: IL-2 (r= 0.613, P value=0.001), IL-4 (r=0.769, P value=0.000), IL-6 (r=0.428 , P value=0.029), IL-8 (r=0.575, P value=0.002), IL-10 (r=0.443, P value=0.023) and IL-13 (r=0.557, P value=0.003). Neither VEGF A nor PIGF were associated with an increase in any other angiogenic analytes. Significant correlations exist between many of the inflammatory analytes, particularly those sharing a common inflammatory cascade. IL-2 correlated strongly with IL-4, IL-6, IL-8, IL-10 and IL-13. Many other significant associations exist between these interleukins. VEGF C displayed a positive correlation with VEGF D (r=0.598, P value=0.001) and Tie 2 (r=0.591, P value=0.001).
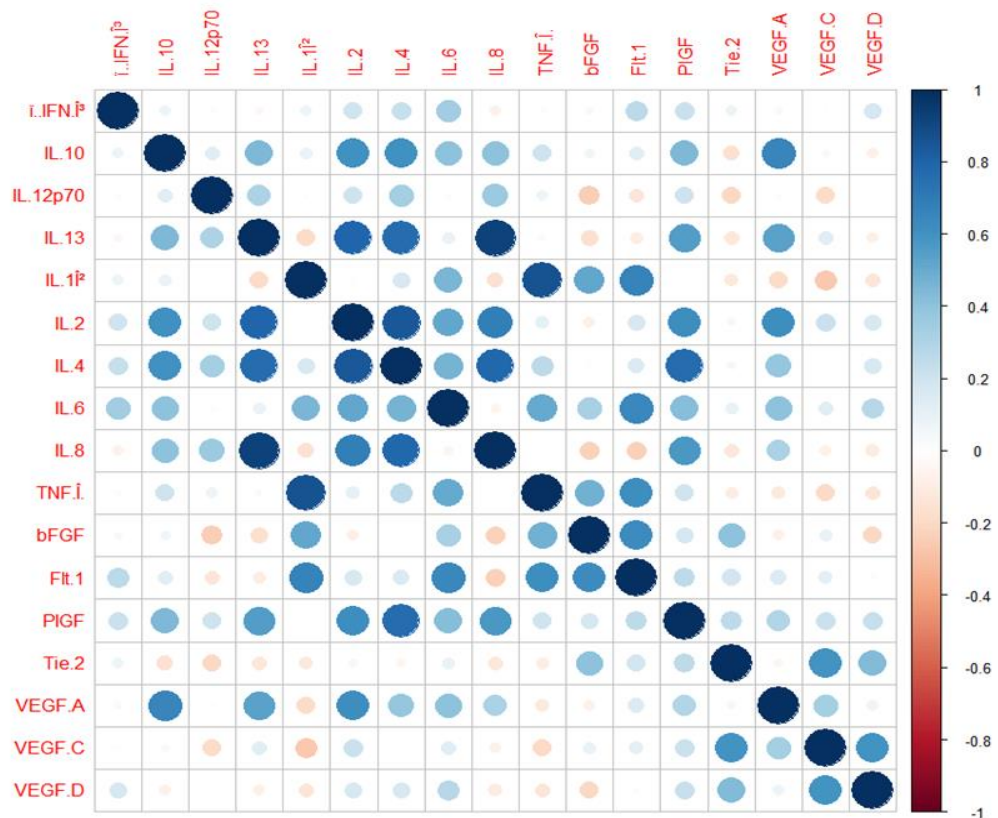
Figure 3.5 *Corrplot of inflammatory and angiogenic analytes.*
TNF-α correlated with Flt-1, bFGF, IL-1β and IL-6. VEGF A secretion correlated positively with IL-2, IL-6, IL-10 and IL-13.

3.9 Correlations between clinical parameters and analyte expression

To evaluate the correlation between clinical parameters and analyte expression corrplots were generated. These corrplots illustrate the relationship between laboratory findings and analyte expression (Figure 3.6), clinical scores and inflammatory analyte (Figure 3.7) and clinical scores and angiogenic analyte (Figure 3.8). CRP correlated significantly with TNF-α (r=0.603, P value=0.001), IL-1β (r=0.625, P value=0.001) and Flt-1 (r=0.492, P value=0.011). Table 3.2 displays all correlations relating to CRP. Albumin displayed an inverse relationship with the same key analytes

but the relationship wasn't as strong; TNF-α (r=-0.585, P value=0.002), IL-1β (r=-0.502, P value=0.009) and Flt-1 (r=-0.438, P value=0.025). Faecal calprotectin did not correlate significantly with any of clinical scores or analytes (Figure 3.6).
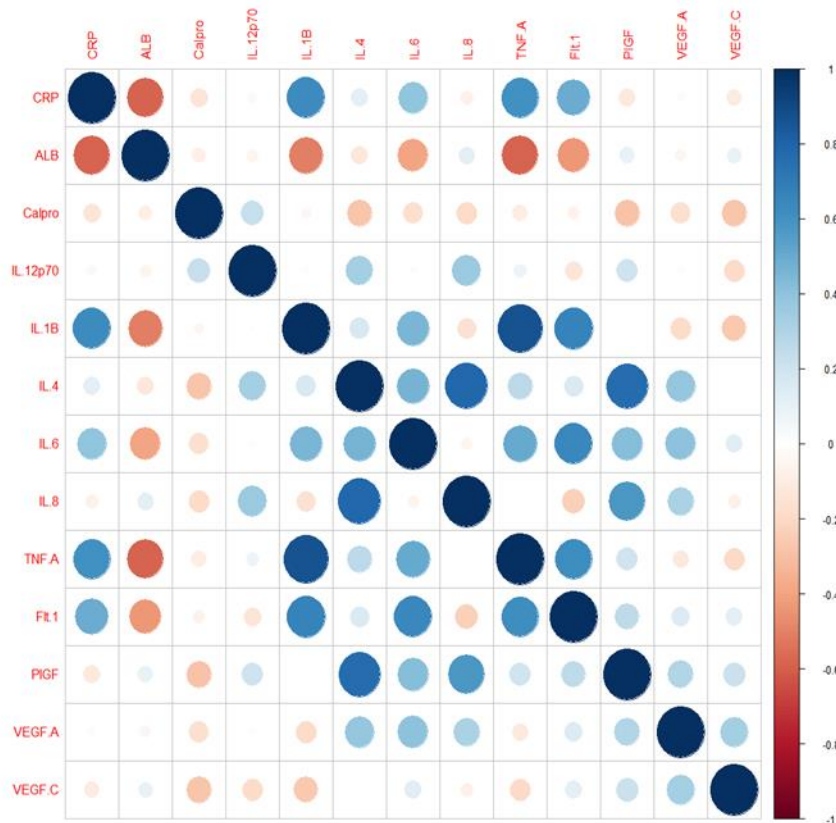
Figure 3.6 *Corrplot with correlations of laboratory parameters and analyte expression.* CRP correlates significantly with TNF-α, IL-1β and Flt-1.

Table 3.2 _CRP Correlations._

This table outlines all clinical, laboratory and analyte correlations with CRP. Statistically significant correlations highlighted in yellow.

Clinical parameter/Analyte	Pearson	P-Value	Number of pairs
ESR	.913**	0.000	23
Bleeding	.859**	0.000	26
Severity	.635**	0.000	26
IL-1β	.625**	0.001	26
TNF-α	.603**	0.001	26
ALB	-.585**	0.002	26
Ulcers	.574**	0.002	26
Endoscopic Mayo	.543**	0.004	26
Hospitalisation	.534**	0.005	26
Total Mayo	.526**	0.006	26
Flt-1	.492*	0.011	26
Hb	-.478*	0.013	26
Clinical Mayo	.469*	0.016	26
Vascular pattern	.451*	0.021	26
Biologic	.445*	0.023	26
Histo Mild/Mod/Severe	.425*	0.031	26
IL-6	.391*	0.048	26
bFGF	0.265	0.191	26
Tie-2	-0.236	0.245	26
Inflam Histo	0.232	0.254	26
Steroid	0.232	0.254	26
Duration >10years	-0.215	0.293	26
Pseudopolyps	0.214	0.293	26
IL-2	0.168	0.411	26
5ASA	0.152	0.458	26
Dysplasia	0.141	0.492	26
Disease duration	-0.126	0.541	26
IL-4	0.119	0.563	26
PIGF	-0.116	0.571	26
IFN-γ	-0.112	0.585	26
VEGF D	-0.110	0.591	26
VEGF C	-0.102	0.619	26
IL-8	-0.072	0.726	26
Disease distribution	0.053	0.795	26
IL-12p70	0.035	0.867	26
IL-10	0.028	0.892	26
VEGF A	-0.025	0.902	26
IL-13	0.020	0.923	26
Calpro	-0.015	0.948	21
Gender	0.012	0.954	26
AZA	-0.008	0.970	26
CRP	1		26

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

3.10 Clinical and histological correlations with analyte expression

Partial Mayo score was found to correlate positively with IL-6 (r=0.517, P value=0.007).

The presence of ulcers at endoscopy were associated with an increased IL-12p70

(Figure 3.7).

Increasing disease duration displayed a signal towards an inverse relationship with

partial Mayo score and inflammatory analytes (TNF- α, IL-2, IL-4, IL-6, IL-8, IL-10, IL-

12p70 and IL-13) however none of these reached clinical significance. When evaluating

the angiogenic panel (Figure 3.8) disease duration was associated with increased Tie-2

(r=0.621, P value=0.001) and VEGF C (r=0.395, P value=0.046). Partial Mayo score

correlated significantly with VEGF receptor (Flt-1) expression (r=0.620, P value=0.001).
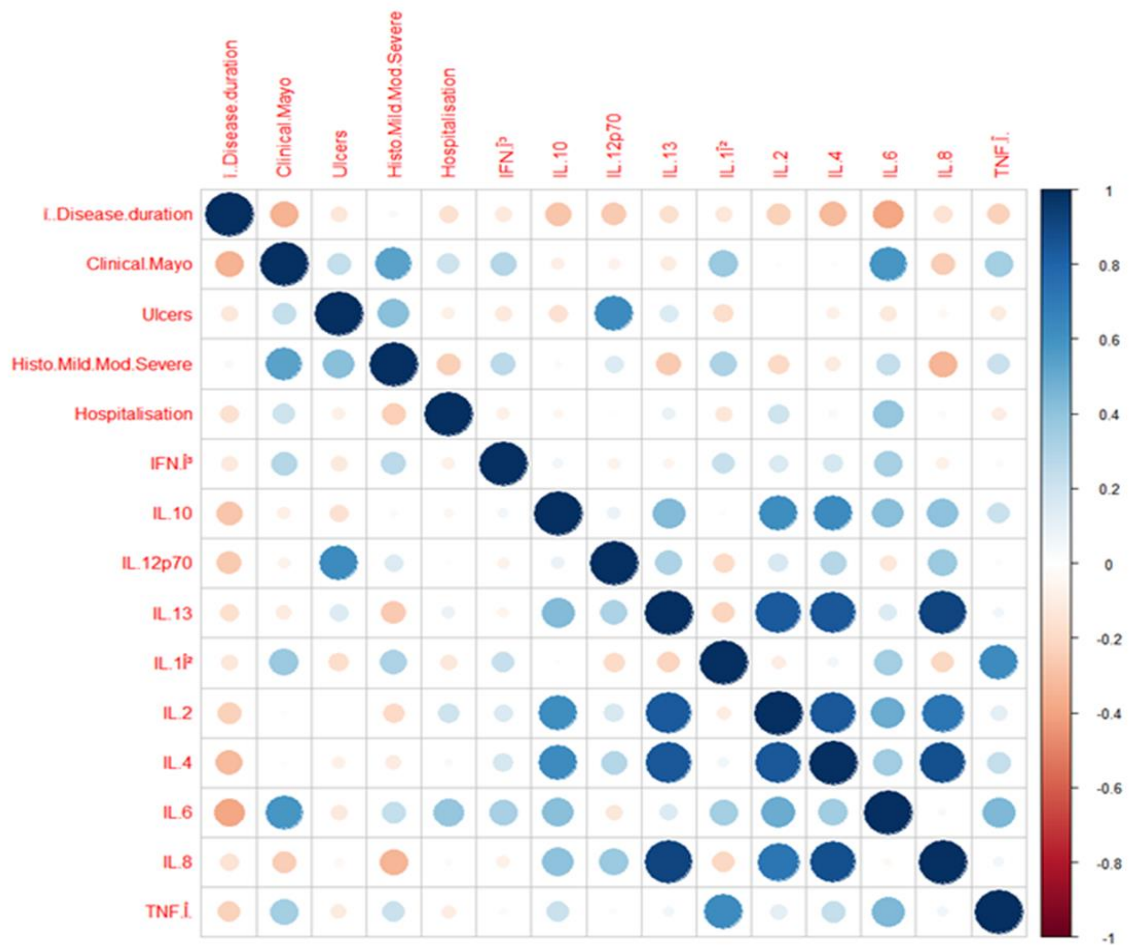
Figure 3.7 *Corrplot of clinical parameters and inflammatory analytes.*
Clinical Mayo score correlates significantly with IL-6. The presence of ulcers at
endoscopy is associated with increased IL-12p70.
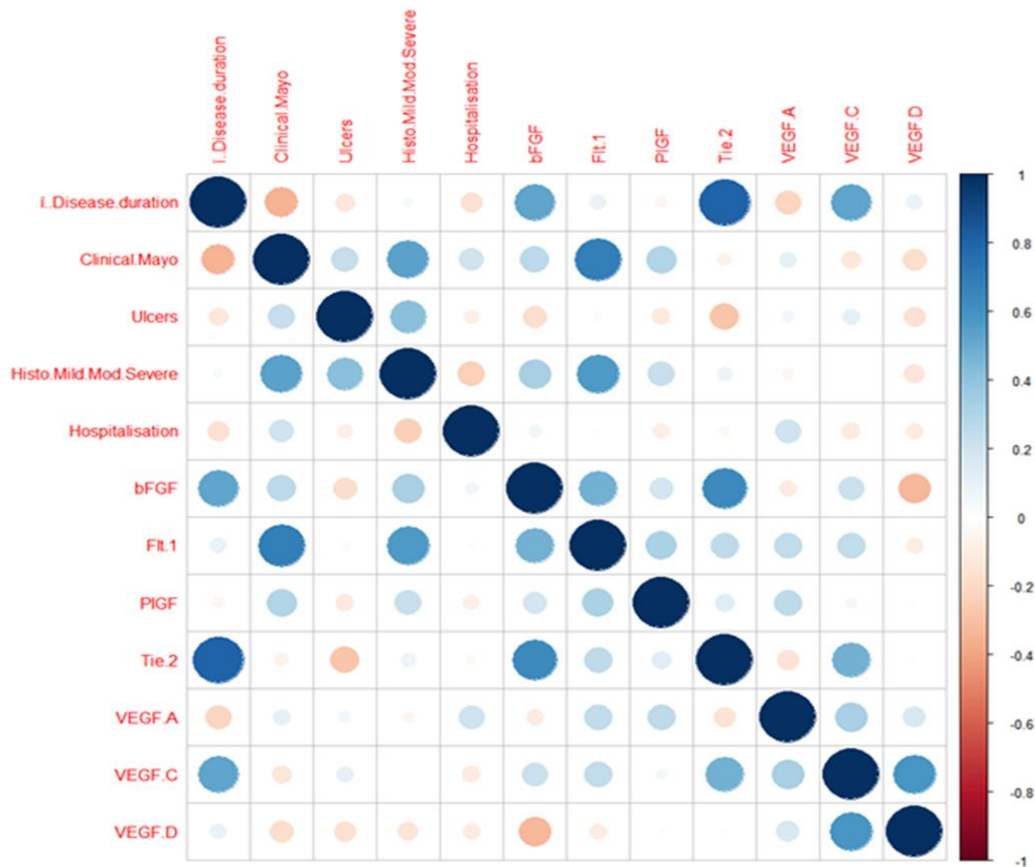
Figure 3.8 *Corrplot of clinical and angiogenic parameters.*
Increased disease duration was associated with an elevated TIE-2 and VEGF C. Clinical
Mayo score correlated with Flt-1 expression.

3.11 Disease duration and analyte expression

The patient cohort was divided by disease duration into two groups (<10 years,

>10years since diagnosis). Patients with shorter disease duration tended to have a

higher Mayo score, although the difference was not significant. An analysis was carried

out to compare analyte expression in both groups (Figure 3.9). Patients with a shorter

duration of disease had higher IL-12p70 compared with longstanding UC patients (P

value= 0.0310). The median readings across all other inflammatory analytes was

higher in those patients with UC <10years, but not statistically significant. There were

no significant differences between the two groups in angiogenic analyte expression.

IFN-γ

IFN-γ
(pg/mL per ug/mL protein)

<10 Years   > 10 Years
Disease Duration

IL-10

IL-10
(pg/mL per ug/mL protein)

<10 Years   > 10 Years
Disease Duration

IL-12p70

*

IL-12p70
(pg/mL per ug/mL protein)

P Value=0.0310

<10 Years   > 10 Years
Disease Duration

IL-13

IL-13
(pg/mL per ug/mL protein)

<10 Years   > 10 Years
Disease Duration

IL-1β

IL-1β
(pg/mL per ug/mL protein)

<10 Years   > 10 Years
Disease Duration

IL-2

IL-2
(pg/mL per ug/mL protein)

<10 Years   > 10 Years
Disease Duration

IL-4

IL-4
(pg/mL per ug/mL protein)

<10 Years   > 10 Years
Disease Duration

IL-6

IL-6
(pg/mL per ug/mL protein)

<10 Years   > 10 Years
Disease Duration

Figure 3.9 *Inflammatory analyte expression in patients with UC for >10years or < 10years.*

Patients with UC <10years had significantly higher IL-12p70. Mann Whitney U test. Line denotes median.
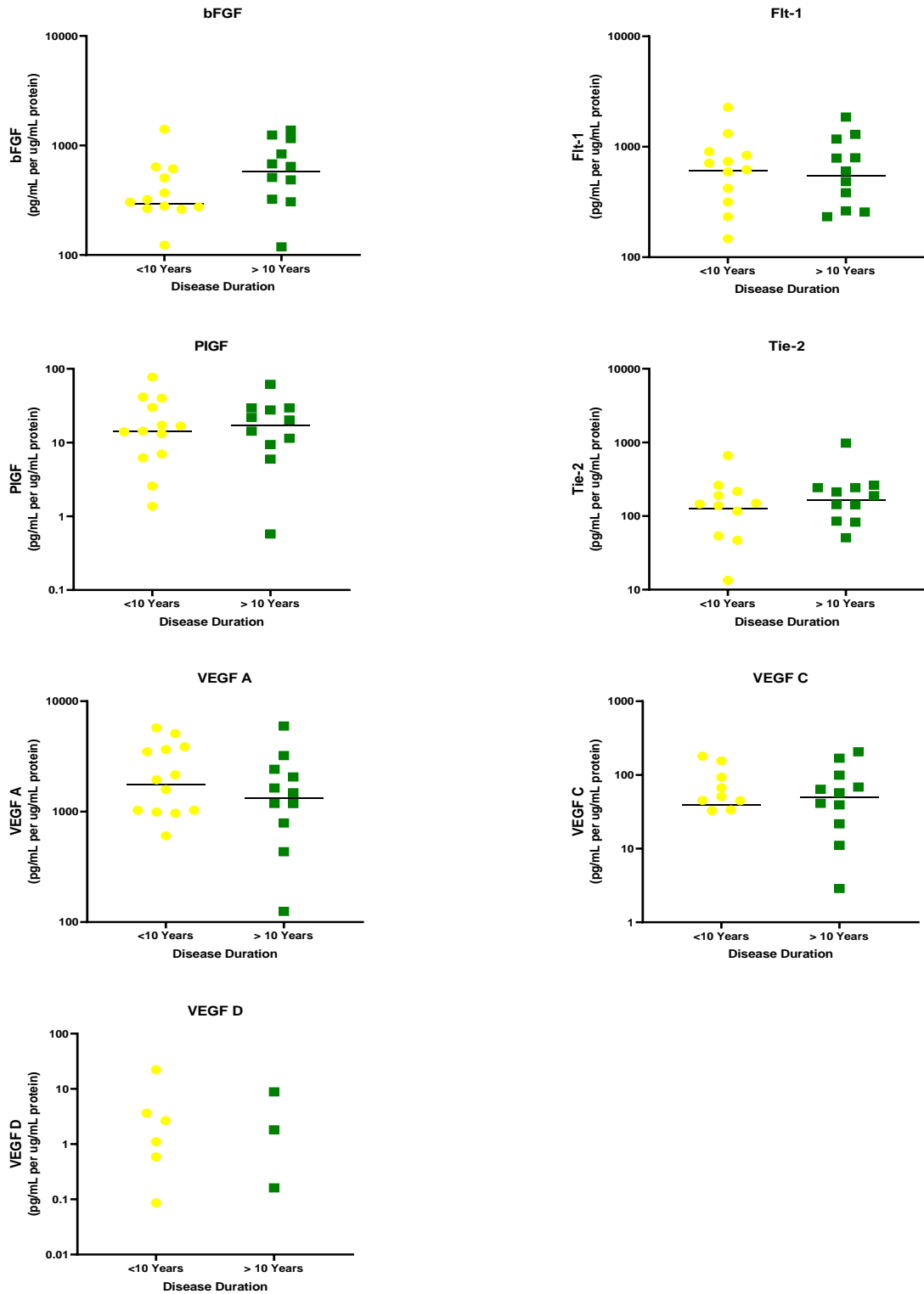
Figure 3.10 *Angiogenic analyte expression in patients with UC for >10years or < 10years.*

Patients with UC <10years had significantly higher IL-12p70. Mann Whitney U test. Line denotes median.

3.12 The effects of histological inflammation on analyte expression

Each patient had colonic biopsies that were assessed by a consultant histopathologist with special interest in gastrointestinal disease. The samples were described as inflamed or non-inflamed based on the presence or absence of architectural changes, crypt destruction, basal plasmacytosis, erosions or ulceration. An analysis was performed to compare analyte expression in patients with inflammation to those without. 11 patients had histology without any evidence of active inflammation, 15 had inflammation of various degrees of severity. On the inflammatory panel (Figure 3.11), IL-1β was significantly higher in the patient cohort with inflammation on histology (P value=0.0362). However, IL-8 was significantly higher in those with non-inflamed histology (P value=0.0204). Of the angiogenic analytes (Figure 3.12), Flt-1 was higher in the inflamed cohort (P value=0.0175).
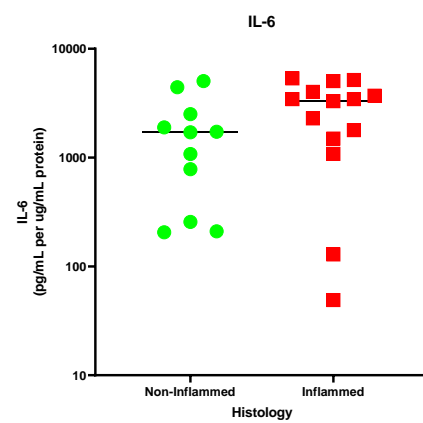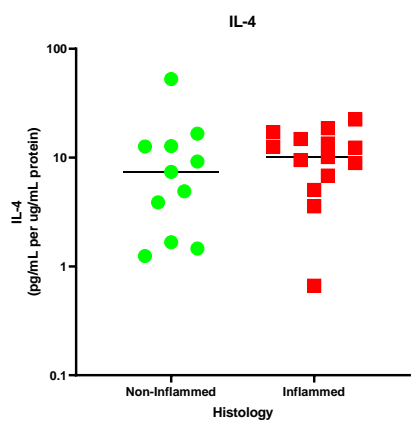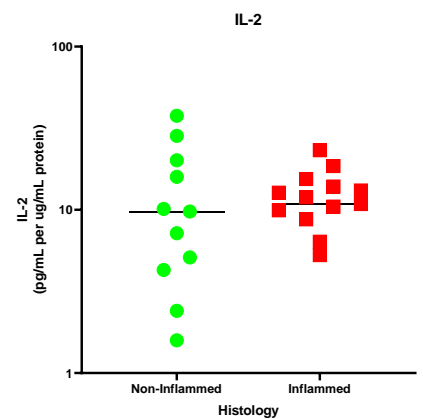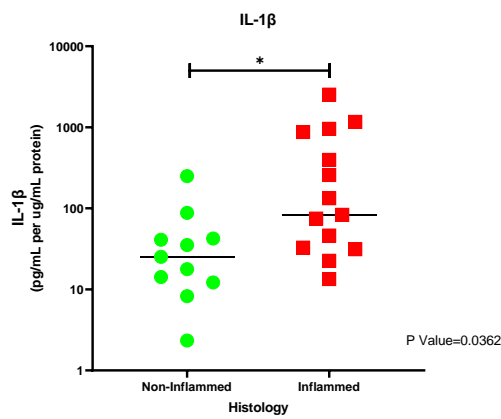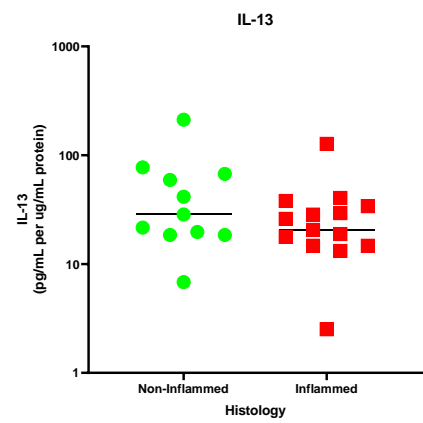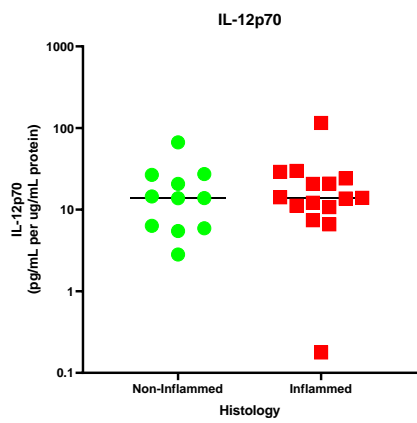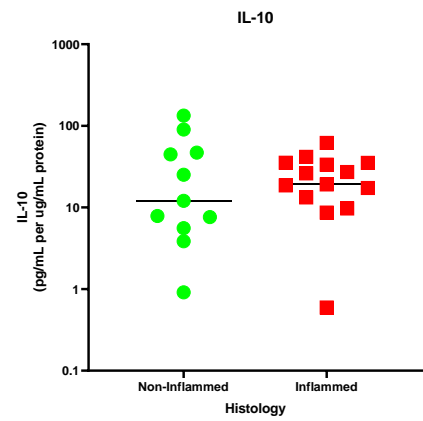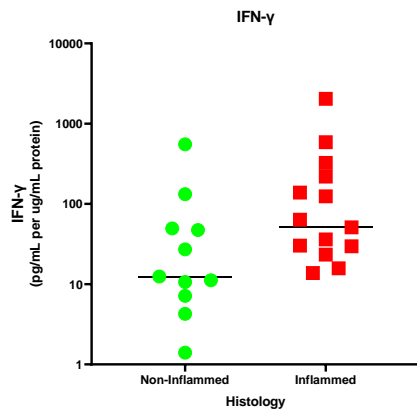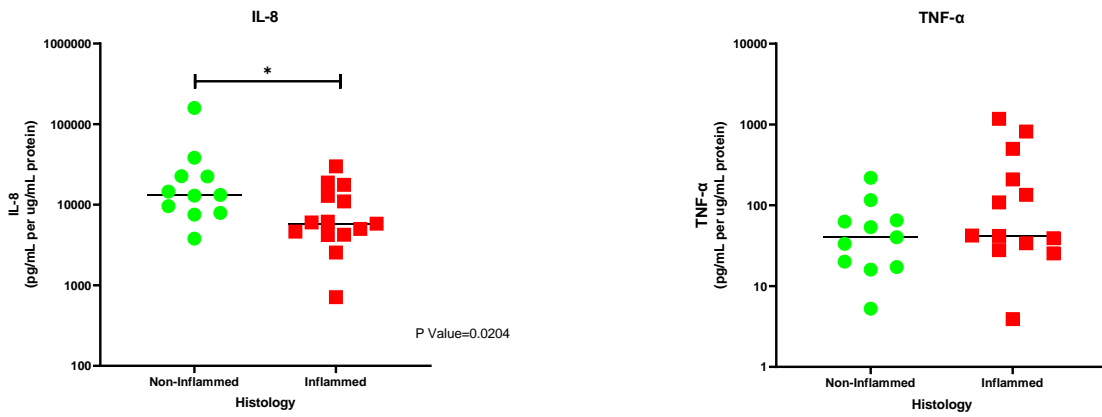
Figure 3.11 *Inflammatory analyte expression in inflamed vs non-inflamed patient cohorts.*

IL-1β is higher in the inflamed patient group, IL-8 is higher in the non-inflamed patient group. Mann Whitney U test. Line denotes median.
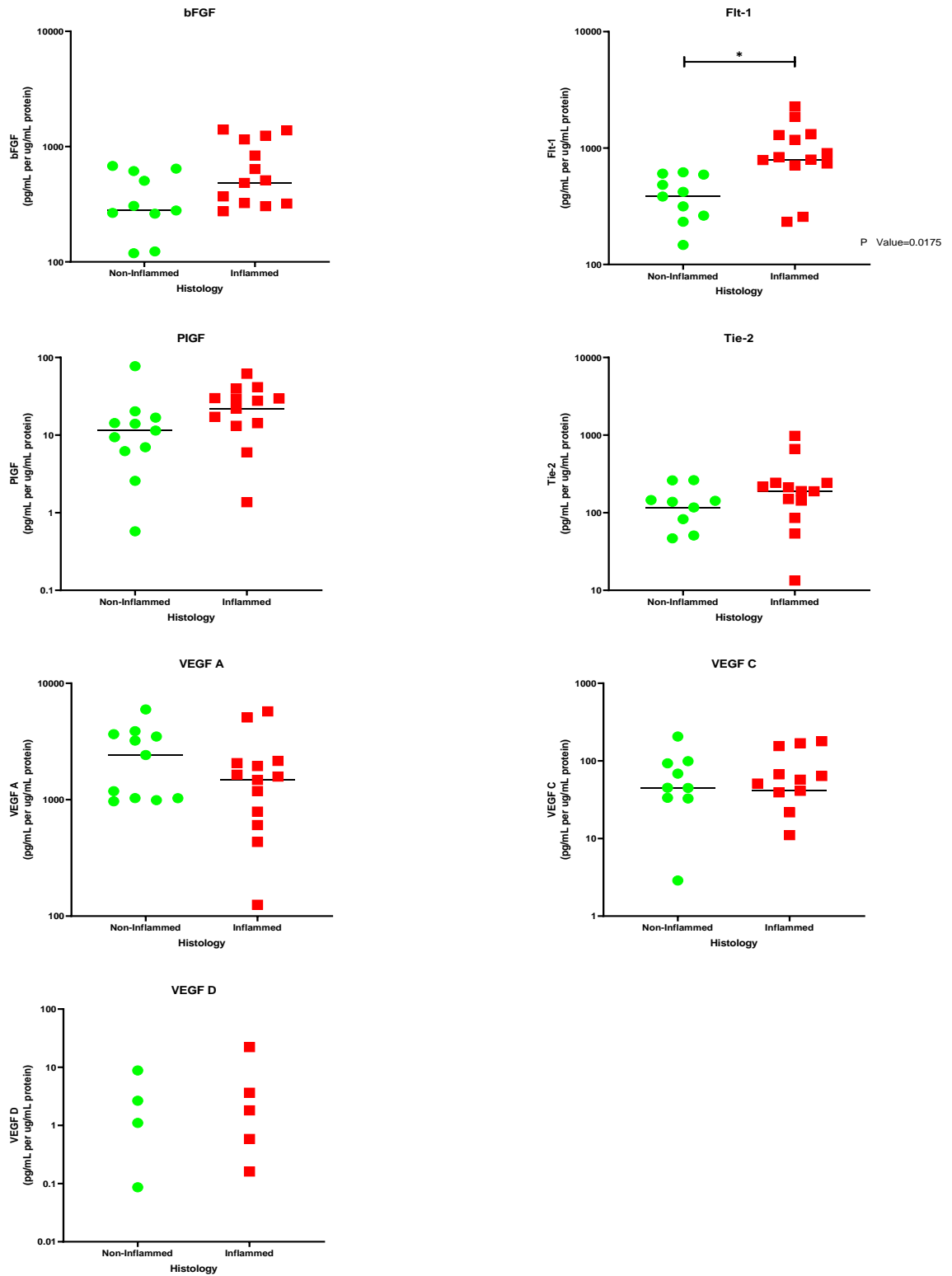
Figure 3.12 *Angiogenic analyte expression in inflamed vs non-inflamed patient cohorts.* Those with inflammation on histology had a higher expression of Flt-1. Mann Whitney U test. Line denotes median.

3.13 Mayo score association with analyte release

The Mayo score for assessing UC activity is an important tool that is commonly used in both clinical practice and research. In our patient group 11 were in remission (score of 0-2), 5 had mild disease activity (score of 3-5) and 10 had moderate disease severity (score of 5-10). An analysis was performed to compare these three patient groups and evaluate the ability of the Mayo score to predict analyte secretion (Figure 3.13 and 3.14). IL-2 and FLT-1 were found to be at significantly higher levels in patients with moderate disease activity compared to those with mild disease or in remission.

IFN-γ

IL-10

IL-12p70

IL-13

IL-1ß

IL-2

P Value=0.0387

IL-4

IL-6
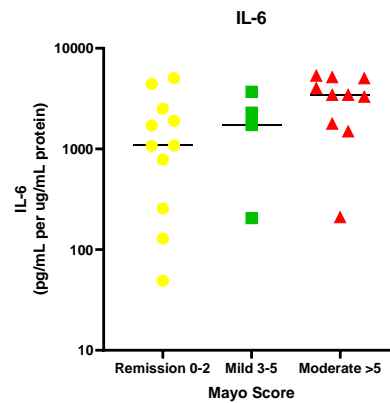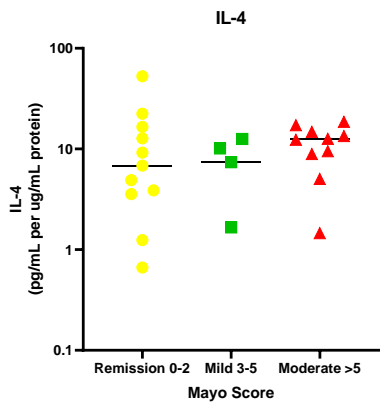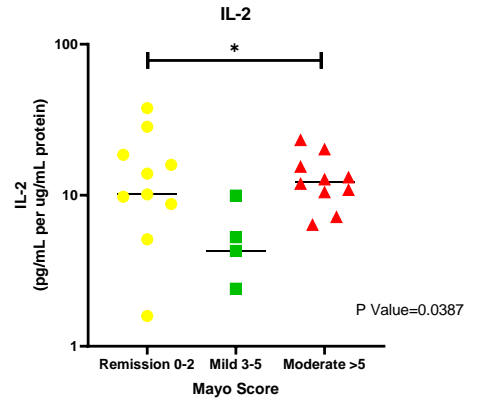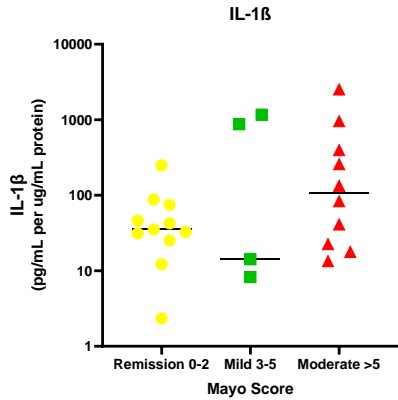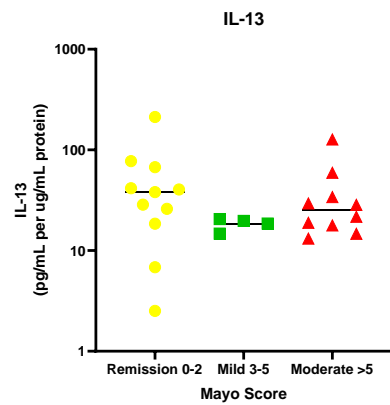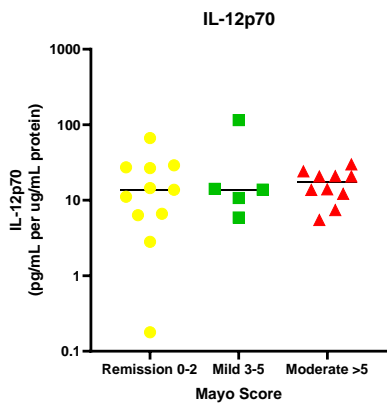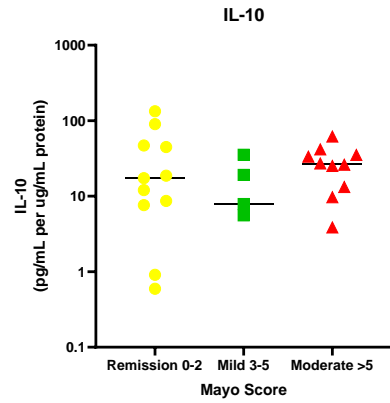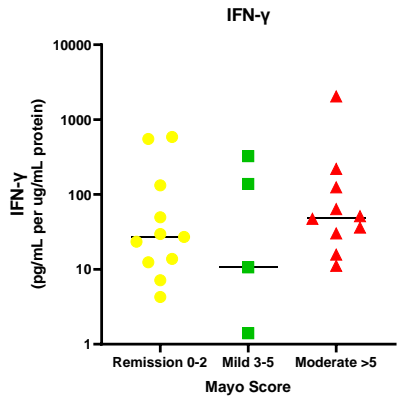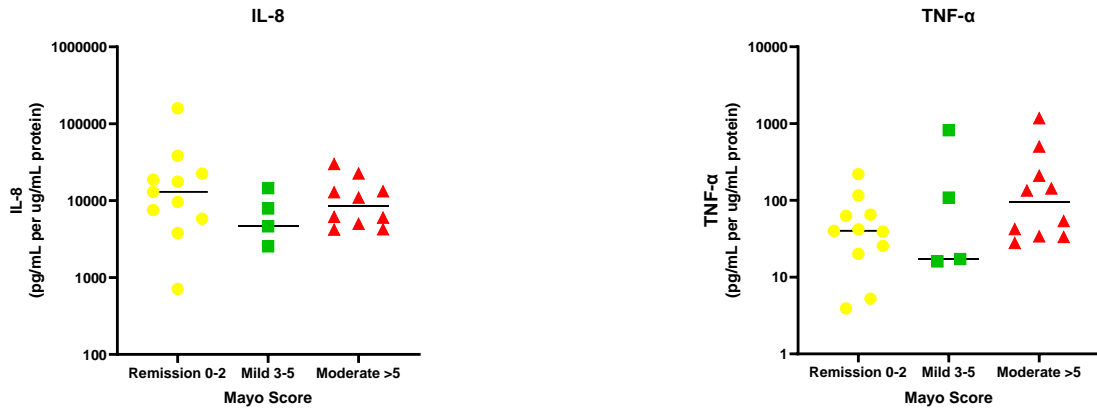
Figure 3.13 *Inflammatory analyte expression in patients in remission (yellow), or with mild (green) or moderate (red) UC according to Mayo score.*

IL-2 is higher in patients with moderate disease activity. Line denotes median. Kruskal-Wallis test.
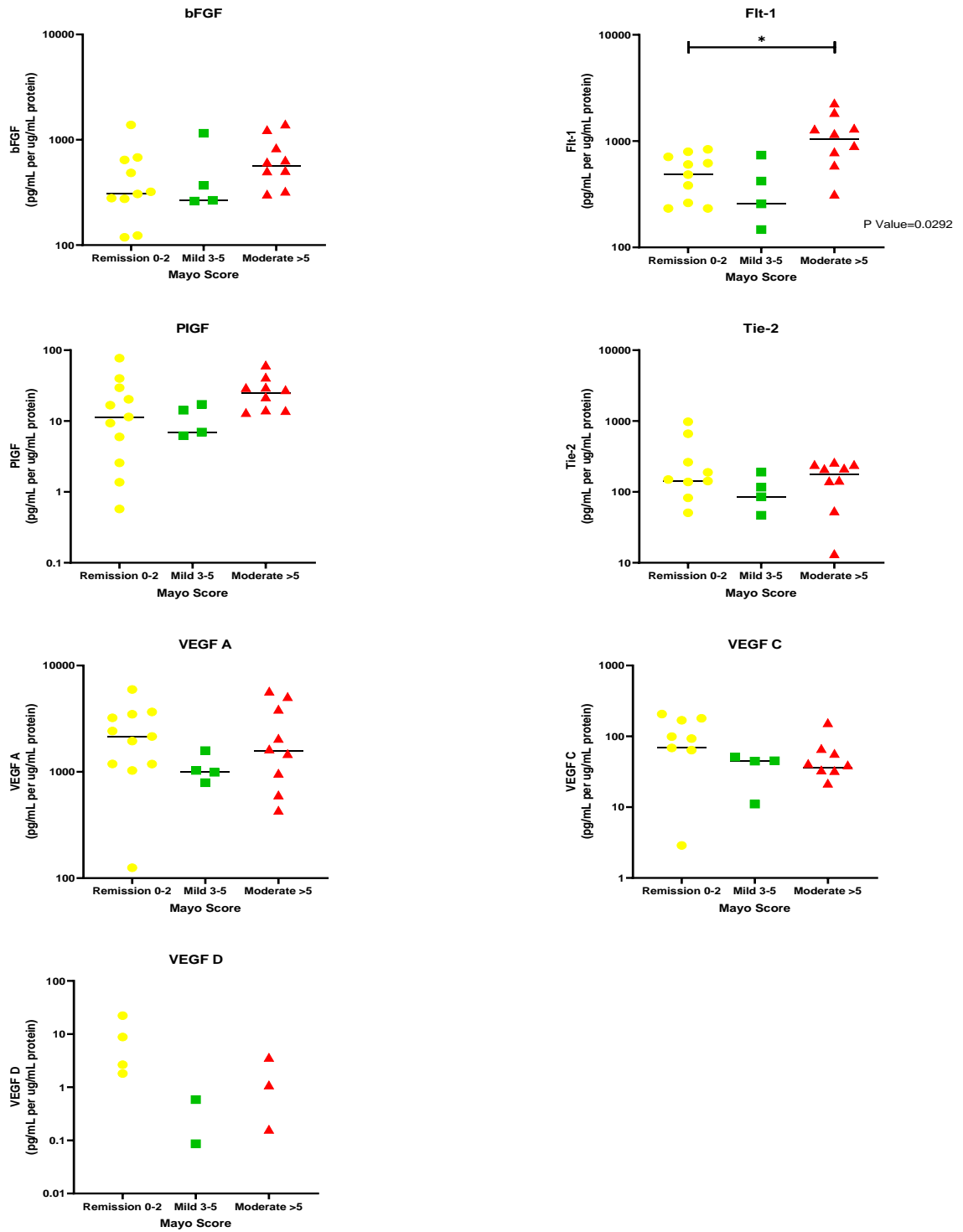
Figure 3.14 *Angiogenic analyte expression in patients in remission (yellow), or with mild (green) or moderate (red) UC according to Mayo score.*
Flt-1 is higher amongst those with moderate disease activity. Line denotes median. Kruskal-Wallis test.

3.14 The effect of biologic exposure on analyte secretion in the colonic microenvironment.

Biologic status did not exert a significant effect on the colonic microenvironment in our patient cohort. In total 9 patients were on active biologic therapy of various types (infliximab, adalimumab, ustekinumab, vedolizumab and golimumab). All 9 patients had at some stage been on an anti-TNF therapy and 5 of them were receiving anti-TNF therapy at the time of the colonoscopy. Patients in the biologic exposed group tended to have a longer disease duration and more severe disease at endoscopy but this was not statistically significant. The biologic naïve group was larger (n=17) with a higher proportion of patients receiving 5-aminosalicylic acid therapy. An analysis was performed on analyte secretion from biologic exposed and biologic naïve patients with no significant differences in expression (Figure 3.15 and 3.16).

IFN-γ

IL-10

IL-12p70

IL-13

IL-8

IL-2

IL-4

Il-6
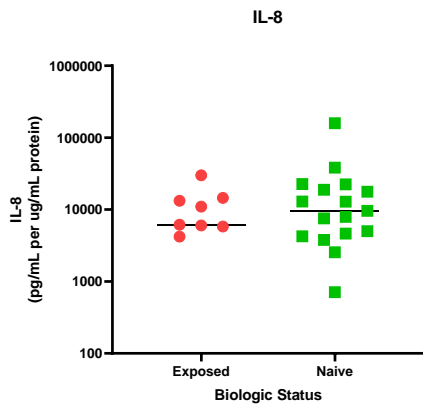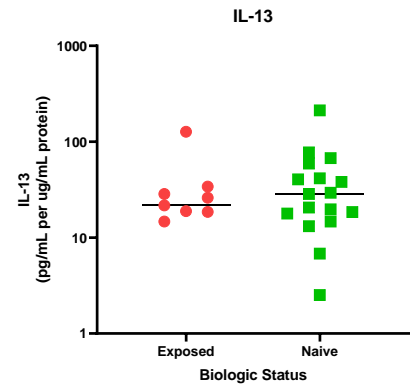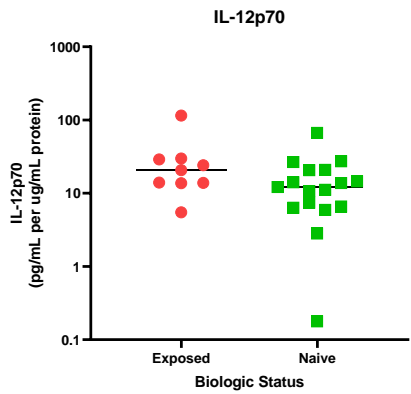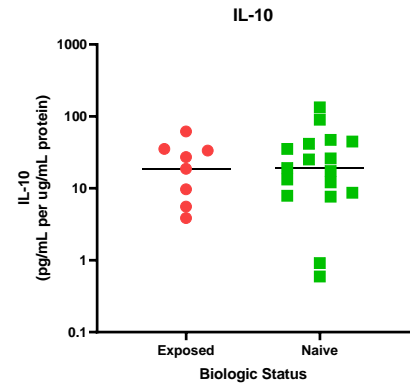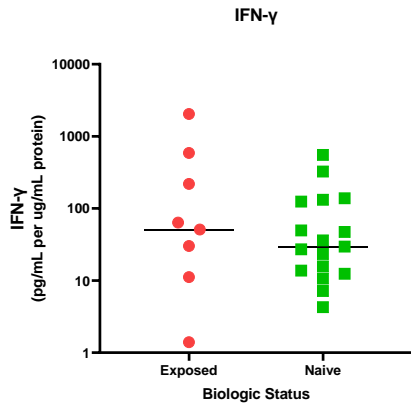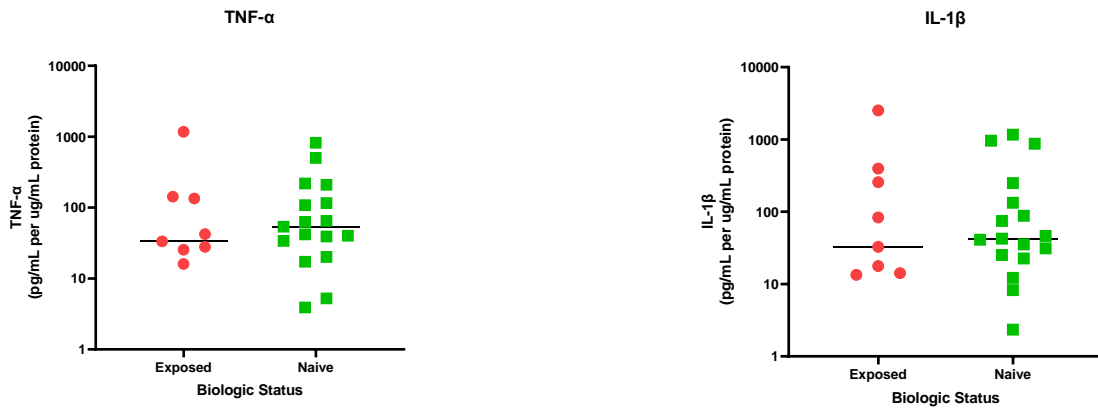
Figure 3.15 *Inflammatory analyte expression in biologic exposed vs biologic naïve patients.*

No significant differences were found when biologic exposed and naïve patients were compared. Line denotes media. Mann Whitney U Test.
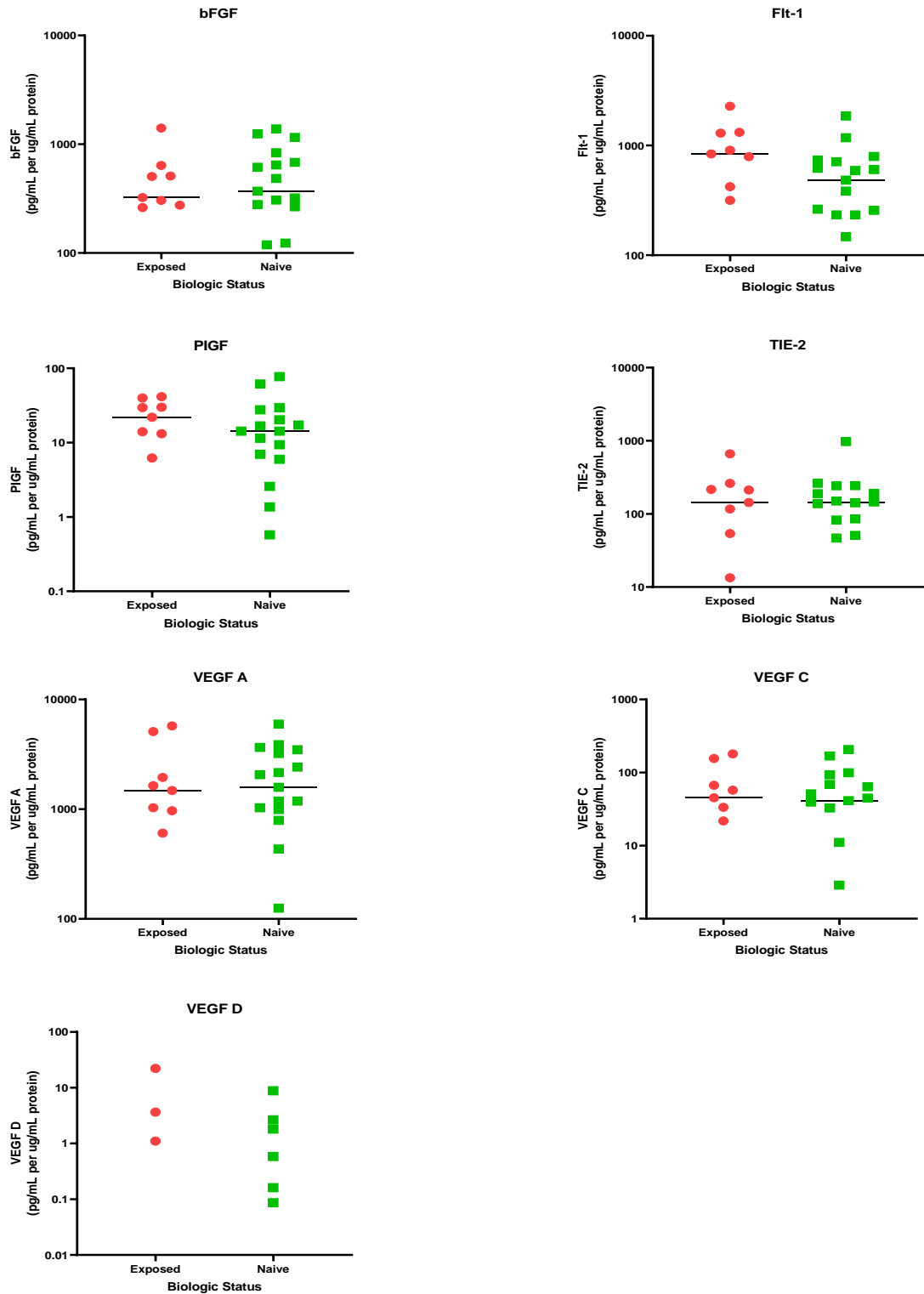
Figure 3.16 *Angiogenic analyte expression in biologic exposed vs biologic naïve patients.*

No significant differences were found when biologic exposed and naïve patients were compared. Line denotes median. Mann Whitney U Test.

3.14 Discussion

3.14.1 Mayo score is an accurate marker of disease severity and correlates with key analytes

This study has shown that the Mayo score is an accurate marker of disease severity. Patients with a high Mayo score were more likely to have ulcers, bleeding and a reduced or absent vascular pattern at endoscopy. A high Mayo score was also associated with increasing levels of inflammation identified at histopathological assessment. There was significant correlation between Mayo sub-scores; patients that had a higher partial Mayo score had a higher endoscopic Mayo score. Many clinical parameters displayed significant positive correlations with one and other. This study lends further support to the use of the Mayo score in clinical practice and research.

This analysis found a significant correlation between clinical Mayo score and IL-6. IL-6 is a pleiotropic cytokine that regulates immunity, it has an important role in chronic intestinal inflammation. It is responsible for perpetuating colonic inflammation in UC and can lead to cancer development by intracellular signal transducer and activator of transcription 3 (STAT3) (106). In another study, IL-6 suppression by anti-TNF therapy was associated with improved clinical outcomes (107).

3.14.2 Inflammatory analytes subside in patients with longstanding ulcerative colitis, but angiogenic markers persist

In longstanding UC the persistent inflammatory changes can sometimes burn out and give way to chronic scarring and fibrotic changes (108). This phenomenon is more

commonly seen in Crohn's disease (109). In our patient cohort those with longstanding

UC had lower levels of inflammatory analytes. Increased disease duration was

inversely associated with inflammatory analyte secretion and a reduced clinical Mayo

score, although this did not reach clinical significance. Patients with increased disease

duration were less likely to be on steroid therapy.

We further evaluated this by comparing patients with UC for >10 years to those with

UC for <10years. There was a statistically significant difference in IL-12p70 with

increased expression in those with shorter disease duration. The median analyte

expression across all other inflammatory analytes was increased in those with UC for

<10years but did not reach significance. Interestingly, increased disease duration was

associated with increased Tie-2 and VEGF C. With increased disease duration the

active inflammation burns out, but pathological angiogenesis persists leading to

diseased, deformed and scarred colon. This may also explain the increased risk of

developing CRC in patients with longstanding UC.

3.14.3 CRP predicts disease severity and correlates with increased TNF-α

In this study CRP was found to be a very accurate marker of disease severity,

corresponding positively with Mayo score. CRP was also associated with reduced

vascular markings, ulcers, bleeding and hospital admission. We sought to confirm this

by performing an analysis on clinical scores in a high and low CRP patient group. Again,

the partial and total Mayo score was significantly higher in those with increased CRP.

Severity at endoscopy was also higher if CRP was elevated. Other studies have had

similar findings, although many different scoring systems have been used to assess UC severity. A prospective study published in 2011 showed significant correlation between CRP and clinical/endoscopic disease activity. In this study disease activity index and the Rachmilewitz score was used to assess UC activity (97). Our findings are consistent with current research and add to the evidence that supports the use of CRP as a non-invasive means of assessing disease activity. We found a negative correlation between CRP and albumin which is explained by the role of albumin as an inverse marker of inflammation. There is a significant body of evidence supporting an inverse relationship between CRP and albumin, particularly in cardiovascular and renal disease (110). In this study patients with a high CRP had a significantly higher erythrocyte sedimentation rate (ESR). ESR is another marker of inflammation, its utility has been proven in UC and other inflammatory conditions. However when evaluated head-to-head in a paediatric population with UC, CRP outperforms ESR, they felt there was little evidence for performing both tests and advised that CRP was superior (98). Our findings support this, although the evidence of a correlation between ESR and CRP is strong, ESR doesn't perform as well at predicting disease severity.

CRP correlated with key inflammatory analytes TNF-α, IL-1β and Flt-1. TNF-α is a target for many of the newer therapies available for UC and plays a key role in disease pathogenesis. CRP is produced primarily by hepatocytes and is activated by TNF-α, interleukin 6 and interleukin 1β (111). It therefore stands to reason that there would be correlation between the two. Despite this there has been very little research evaluating the relationship between these two molecules. This relationship has been

studied in different settings; however very little data exists. In a study of a group of

male patients who were post-operative, plasma levels of leptin, TNF-α and CRP were

recorded. They found that both TNF-α and CRP correlated strongly with leptin levels

(112). They postulated that there was likely correlation between the two, but this was

not formally investigated. Another study has shown evidence that CRP can stimulate

production of TNF-α in alveolar macrophages. Production of TNF-α increased in a dose

dependent manner following stimulation by CRP (113). This significant relationship

between CRP and TNF-α provides a very important insight into the microenvironment

in UC. Response to anti-TNF therapy in UC is often supported by a reducing CRP, this is

likely due to reduced inflammatory burden, but perhaps this also reflects a reduction

in TNF-α circulating in the inflamed colon.

In our study CRP also correlated with IL-1β and VEGF receptor (FLt-1). Interestingly,

when human alveolar macrophages were treated with CRP they stimulated production

of IL-1β as well as TNF-α (113). We have made similar findings, confirming significant

crosstalk between CRP and key inflammatory analytes in UC. The role of CRP in

angiogenesis is unclear, one would hypothesise that a perpetual inflammatory state

will drive angiogenesis and as a result expect an association to exist, however the data

is mixed. There is evidence that CRP is associated with upregulation of VEGF and

increased angiogenesis (114). However, there is also evidence that CRP impairs

angiogenic functions (115). In our analysis CRP was associated with increased

expression of Flt-1, however there was no association with VEGF A.

Albumin was inversely related to TNF-α, IL-1β and Flt-1, intuitively this appears

appropriate given that albumin is an inverse marker of inflammation. The correlations

are not as strong as those found with CRP. In other studies serum albumin has been

shown to be an important factor in predicting response to anti-TNF therapy. Patients

with higher serum albumin levels achieved higher drug levels and had improved

clinical outcomes (100). Increased intestinal inflammation is associated with increased

colonic protein loss, this may also explain the role of albumin as a marker of disease

severity (116).

3.14.4 Significant crosstalk between inflammatory and angiogenic analytes

TNF-α secretion correlates with basic fibroblast growth factor and VEGF receptor 1

TNF-α is a key inflammatory analyte in UC pathogenesis and therapeutics. In this

analysis a positive correlation between TNF-α, FLT-1 and bFGF was found. To date

there has been little research into the role of TNF-α in angiogenesis. TNF-α is thought

to display both anti- and pro-angiogenic activity and is context dependent. Data exists

that suggests TNF-α exerts an anti-angiogenic effect through modulation of FLT-1 and

KDR/FLT-1 (117). However, there is conflicting evidence that shows TNF-α has a pro-

angiogenic effect. In a murine study increased TNF-α was associated with an increased

expression of VEGF and its receptors (FLT-1) (118). In human glioma cells a strong

positive relationship was noted between TNF-α expression and the secretion of VEGF,

FLT-1, IL-1β and bFGF. It was hypothesized that VEGF and FLT-1 release was enhanced

by TNF-α and bFGF through their activity on transcription factor SP-1 (119). Our analysis provides supporting evidence of a strong positive correlation between VEGF receptor and TNF-α. Our data also shows a positive relationship between bFGF expression and TNF-α, this has been studied and prior research has highlighted their important inter-dependence. An immunohistochemical study of the mouse cornea revealed increased levels of TNF-α where angiogenesis was occurring, and this was associated with an increase in bFGF. Although conflicting evidence exists the majority of the data support a positive role exerted by TNF-α in stimulating angiogenesis. The mode of action by which this occurs is not fully understood. Therapeutic targeting of TNF-α has provided a breakthrough in the management of many chronic inflammatory conditions, these therapies have been shown to indirectly provide an anti-angiogenic effect. This provides further evidence of the role of TNF-α in stimulating angiogenesis (120). In our analysis we found a positive correlation between TNF-α and key analytes FLT-1 and bFGF supporting the hypothesis that there is significant crosstalk between inflammatory and angiogenic cytokines.

TNF-α also correlated with other inflammatory analytes IL-1β and IL-6, this is to be expected given TNF-α works directly on the cytokine cascade of which these analytes are a part. The relationship between TNF-α and IL-1β and IL-6 has been studied in bone resorption pathophysiology and in neural cells (121, 122). These 3 cytokines are closely related, and this analysis has provided further evidence of their interdependence.

Key angiogenic analyte VEGF A correlates with IL-2, IL-6, IL-10 and IL-13.

Our study revealed a significant correlation between the most potent angiogenic analyte, VEGF A and IL-2, IL-6, IL-10 and IL-13. The relationship between VEGF A and IL-2 has been previously identified, although not fully understood. Suppressing VEGF secretion also leads to a reduction in IL-2. As anti-VEGF therapies have become more commonplace a study was conducted in patients with renal cell carcinoma who had IL-2 supplementation following VEGF suppression. However, severe cardiac toxicity was noted in those receiving IL-2 and the study was discontinued (123). Our study supports the evidence that a relationship between VEGF A and IL-2 exists. Similarly, there is strong evidence that IL-6 plays a role in VEGF dependent angiogenesis. IL-6 has been shown to increase cervical tumour growth via STAT3 pathway through VEGF dependent angiogenesis (124). A study of patients with breast cancer also found a significant positive correlation between IL-6 and VEGF A (125). VEGF A correlated the most strongly with IL-10. There is conflicting data on the role of IL-10, overall it is thought to have an anti-inflammatory effect (126). However, the role of IL-10 is often determined by specific factors in the microenvironment. The IL-10 regulation of macrophages is context dependent, it inhibits the predominant bone marrow derived macrophages, but does not exert an effect on prostaglandin stimulated macrophages. Furthermore in a study under hypoxic conditions IL-10 did not suppress VEGF release from either macrophage type (127). These findings were reproduced in a study evaluating the role of IL-10 in angiogenesis in the retina, again when tested under hypoxic stimulus, IL-10 not only failed to block, but appeared to stimulate macrophage

migration to the retina and release of VEGF (128). IL-10 is a complex cytokine, overall it appears to have an anti-inflammatory effect, however the relationship with VEGF A is intricate and context dependent. In our analysis we found that a positive correlation existed between IL-10 and VEGF A expression. Our study also found a moderate positive relationship between VEGF A and IL-13. To our knowledge this relationship hasn't been evaluated to date. IL-13 has a similar structure to IL-4 and shares a common receptor. IL-13 is important in goblet cell hyperplasia, mucus secretion and IgE synthesis. It is an important cytokine in asthma as it plays a role in airway hyperresponsiveness and fibrosis. It also exerts an angiogenic effect through chronic inflammation.

Placental growth factor (PIGF) is an important molecule in angiogenesis and vasculogenesis. PIGF is primarily produced in the placental trophoblast but is also produced at lower levels in the lung, thyroid, heart and skeletal muscle. In our analysis we found that PIGF demonstrated a positive correlation with IL-2, IL-4, IL-6, IL-8, IL-10 and IL-13. Very little data exists analysing this relationship, however these findings do suggest significant crosstalk between PIGF and inflammatory analytes. IL-10 has been found to be increased in association with PIGF in early pregnancy (129). Interestingly neither VEGF A nor PIGF correlated with any of the angiogenic analytes. Many angiogenic molecules have a specific receptor and can exert their effect independent of each other. Conversely many of the interleukins are part of a shared inflammatory cascade and as a result expression correlates strongly. IL-2 sometimes called the activator and controller displays significant correlation with many other interleukins. In our study it was associated with increased IL-4, IL-6, IL-8, IL-10 and IL-13.

This analysis has demonstrated significant crosstalk between key inflammatory and angiogenic molecules. TNF-α is an important target in the management of UC, here we have shown a significant positive correlation with VEGF receptor and bFGF. Similarly, VEGF A, the most potent stimulator of angiogenesis correlates positively with IL-2, IL-6, IL-10 and IL-13.

Biologic exposure does not impact on analyte expression in the UC microenvironment

Biologic status did not exert a significant effect on analyte secretion in our patient cohort. 9 patients were actively receiving biologic therapy. Many different therapy types were being delivered with different modes of action. Given the heterogenous combinations of medication being delivered and the small numbers it is difficult to comment definitively on their effect on analyte release.

3.15 Summary of findings:

This chapter adds to the evidence supporting the use of CRP as a non-invasive tool for assessing disease severity in UC. This novel analysis identified a significant correlation between the serum CRP and TNF-α expression within the colonic micro-environment. Although TNF-α is a key cytokine and therapeutic target in UC, its relationship with CRP has not been previously assessed.

In this chapter we discovered significant interrelationships between angiogenic and inflammatory analytes. TNF-α correlated significantly with important angiogenic analytes FLT-1 and bFGF. VEGF A the most potent angiogenic molecule was associated with increased IL-2, IL-6, IL-10 and IL-13.

The partial Mayo score correlated with increased IL-6 and FLT-1 expression. Interestingly we demonstrated that increased disease duration was associated with increased angiogenic molecule expression (TIE-2 and VEGF C), and a non-significant signal indicating an inverse relationship with inflammatory analyte expression.

Profiling our patient cohort's clinical details and comparing with analyte expression from the colonic microenvironment has provided a novel insight into UC pathogenesis. Our study supports the use of clinical scoring systems and laboratory findings in assessing disease activity. This novel analysis reveals crosstalk between key angiogenic and inflammatory analytes within the colonic mucosa. The clinical relevance of these analytes have been closely examined.

Chapter 4:

Therapeutic targeting of ulcerative colitis explants with novel anti-angiogenic and anti-inflammatory small molecule agents

4.1 Introduction

In this chapter the effects of anti-inflammatory and anti-angiogenic small molecule agents on the UC colonic microenvironment will be evaluated. These agents have the potential to unlock a novel therapeutic approach in the management of this chronic inflammatory condition. Recent breakthroughs have changed the disease course in patients with UC, however many still have poor outcomes. This is a timely research project that has the potential to result in an improved quality of life for sufferers of UC.

When first described in the late 19th century UC carried a significant mortality. A patient presenting to a hospital with severe UC 80 years ago would have had a mortality of up to 60% (130). Treatments at this time consisted of high protein intake, low residue diet, bed rest and vitamin supplements. Topical therapies such as silver nitrate, hydrogen peroxide and bismuth were prescribed, evidence for these treatments were anecdotal and outcomes were often poor. Patients frequently suffered peritonitis, major haemorrhage and sepsis. The different surgical approaches employed historically, varied significantly, many technical problems and complications were encountered with patient's symptoms left unresolved. In 1952 surgeon Bryan Brookes modified the ileostomy to good effect (131). Surgery in the form of the proctocolectomy provided a curative option for patients by the late 1950s. Despite this advance, mortality remained high, patients were often reluctant to undergo surgery or were critically unwell by the time they did.

The first effective medical therapy for UC was sulphasalazine. Its mechanism of action is not fully understood but thought to be related to inhibition of prostaglandins. This inhibition results in a local anti-inflammatory effect. Sulphasalazine has been approved for use in UC since the 1950s. Despite the introduction of sulphasalazine, outcomes for patients with severe UC remained poor. The true revolution in the management of UC came with the advent of corticosteroids. A landmark study in 1955 showed that in patients with severe UC corticosteroids reduced mortality from 24% to 7% (132). They also achieved much higher rates of remission when compared with control (42% vs 13%). Earlier surgery became a feature in the corticosteroid era. If patients were failing steroid therapy by day 5 surgery was recommended. Mortality was significantly lower in patients who received surgery early after failed steroid therapy (1 vs 11%) (133).

In the early 1990s the concept of rescue therapy was developed. This involved treatment of those failing steroid therapy to avoid surgery. The two main drugs used were cyclosporine and later infliximab. Cyclosporine, a calcineurin inhibitor is administered via intravenous infusion. Cyclosporine is effective at inducing remission in severe UC, but cannot be used as a maintenance therapy. Azathioprine, another immunosuppressant, was used to maintain remission. Concerns exist regarding toxicity of azathioprine, side effects such as renal failure, hypertension, hepatoxicity and diabetes have been reported (134). Analysis of the pre and post cyclosporine era showed that the colectomy rate had remained stable. Although emergency surgery had been avoided for many, most went on to require colectomy eventually (135). Monoclonal antibody, infliximab, has been shown to be effective at inducing and maintaining remission in UC. The ACT trials showed a significant clinical response to

infliximab therapy compared with placebo at weeks 8, 30 and 54 (136). Infliximab appears to have a more favourable side effect profile than cyclosporine and can be used as a maintenance treatment (137).

In more recent years there have been a plethora of biologic agents approved for use in UC. Adalimumab is a humanized monoclonal antibody that like infliximab binds to TNF-α. It is delivered via a subcutaneous injection and is therefore more convenient for patients. It allows patients to take a more active role in management of their UC, however compliance can be an issue. Two large double blinded randomized phase 3 clinical trials were undertaken to evaluate the role of adalimumab at inducing and maintaining remission (ULTRA 1 and 2). In ULTRA 1 patients enrolled were naive to biologic therapy, ULTRA 2 included patients with prior biologic exposure. In both trials remission rates were significantly higher in the adalimumab (160/80mg) group when compared with the placebo group. In the subgroup with prior biologic exposure rates of remission were lower but still outperformed placebo (138). Golimumab is another human monoclonal antibody that targets TNF-α. It is effective in the management of UC, ankylosing spondylitis, rheumatoid and psoriatic arthritis. Its role in UC was evaluated by the PURSUIT trials. Golimumab achieved a clinical response in over 50% of patients at week 6. In the maintenance therapy arm of the study rates of remission were significantly higher in the golimumab 100mg group but not in the 50mg groups when compared with placebo (139). These three agents have shown the importance TNF-α plays in UC. The therapeutic targeting this inflammatory cytokine has significantly improved patient's symptoms and quality of life.

Vedolizumab is approved for use in UC, it is delivered via intravenous infusion. It is an agent that reduces leukocyte adhesion by binding α4β7 integrin thereby preventing interaction with MAdCAM-1 (140). Because it targets α4β7 integrin vedolizumab is thought to exert a gut specific effect, as a result systemic immunosuppression is avoided. Vedolizumab is favoured in older patients or those with a history of malignancy. Its role in UC was studied in the GEMINI trials. GEMINI included patients who were biologic naïve and exposed. At week 6, the clinical response in the treatment group was 47% vs 25% in the placebo cohort. Rates of clinical remission and colonic mucosal healing were also significantly higher in the vedolizumab group. Vedolizumab was also more effective at maintaining steroid free remission at week 52 than placebo (141).

Tofacitinib is a JAK inhibitor that is used in various inflammatory conditions and to prevent allograft rejection in patients post-transplant. It inhibits JAK 1, 2 and 3 which in turn modulate multiple interleukins. Tofacitinib is delivered via oral tablet. A phase 3 double blinded study evaluated the role of tofacitinib as an induction agent in UC (OCTAVE trial). Patients that were refractory to steroids and biologic agents were included. Clinical response and remission at 8 weeks were measured. Tofacitinib outperformed placebo at the higher doses (32). Tofacitinib appears to be safe and well tolerated, but one case of a perianal abscess was noted in the treatment group. The role of tofacitinib as a maintenance treatment has not yet been investigated.

Ustekinumab blocks the p40 subunit of IL-12 and IL-23. It is delivered initially via intravenous infusion, followed by subcutaneous injection. In the UNIFI trials its role in

induction and maintenance of remission in UC were evaluated. An 8 week induction trial and 44week maintenance trial were undertaken. Clinical remission at week 8 was 15.6% in the treatment group vs 5.3% in the placebo group. The percentage in remission at week 44 was higher in the treatment group 43.8% vs 24% (33). UNIFI reported good tolerability and a similar side effect profile to placebo. However, there were 2 deaths in the treatment group and 7 new cancers diagnosed, vs no deaths and 1 cancer in those who received placebo. Overall the UNIFI trial confirmed that ustekinumab was more effective than placebo at induction and maintenance of remission in UC (33).

Although significant breakthroughs have been made in recent years the overall effectiveness of these newer treatments are in many cases underwhelming. Clinical trials use strict parameters to define remission and this in part explains the relative low percentages of patients who respond to these newer treatments. What is clear is that there is significant need to continue to pursue further advances and new targets in the management of UC. In this chapter the effect of two novel patented small molecule drugs will be evaluated for their ability to suppress the overproduction of inflammatory and angiogenic analytes. With focus on analytes that have been shown to play an important role in UC pathogenesis. TNF-α and IL-12, in particular, have been targeted to good effect by other agents. Targeting angiogenic analytes provide a novel therapeutic approach. Chronic inflammation and pathogenic angiogenesis are inextricably linked. Furthermore, there is a risk of developing CRC in patients with UC. Anti-angiogenic agents are used in the management of CRC, our novel agents may provide a chemopreventative effect.

4.2 Aims of chapter 4

1. Evaluate the ability of pyrazinib (P3) and 1,4-dihydroxy quininib (Q8) to reduce the overproduction of inflammatory and angiogenic analytes compared with control in colonic tissue cultured media (TCM) from a patient cohort with ulcerative colitis.

2. Study the effect of the drugs in various sub-groups to establish if these treatments are more efficacious in certain cohorts. With focus on patients who are biologic naïve vs exposed, high vs normal CRP, inflammation on histology and disease duration.

3. Compare P3 and Q8 to establish which drug is more effective at reducing analyte expression.

4.3 Methods

The methods used are described in detail in chapter 2. Colonic UC biopsies were obtained from patients presenting for colonoscopy to St James's Hospital. The samples were cultured in either P3, Q8 and control. Biopsies were snap frozen in liquid nitrogen. Tissue cultured media was analysed using V-plex pro-inflammatory and angiogenesis MSD multiplex ELISAs. Bicinchoninic acid (BCA) assay was performed to calculate the protein content of each biopsy and normalize analyte expression.

4.4 Results

4.4.1 Pyrazinib (P3) and 1,4-dihydroxy quininib (Q8) alter the secretion of angiogenic and inflammatory molecules

26 patients were enrolled in the study. Colonic biopsies were taken from each patient and cultured in P3, Q8 and control. Both agents resulted in down regulation of inflammatory and angiogenic analytes when compared with control. Table 4.1 shows the overall effect of the drugs on each analyte with statistically significant findings highlighted in yellow. Key analytes TNF-α, IL-12p70 and VEGF-A were significantly suppressed by both agents (Figure 4.1 and 4.2). IL-2, 8 and 13 were also downregulated by both agents. P3 inhibited angiogenic analytes VEGF-C and PIGF. All analytes were reduced when treated with either drug with the exception of VEGF-D which was up-regulated, although non-significantly, by Q8.

Table 4.1 *Effect of Q8 (left) and P3 (right) on inflammatory and angiogenic analytes.* Significant P-values (<0.05) in yellow. Overall effect of the drug indicated by arrow.

Effect Of Q8 on Analyte Expression				Effect Of P3 on Analyte Expression			
ANALYTE	No. of Pairs	P VALUE	REDUCE/STIMULATE	ANALYTE	No. of Pairs	P VALUE	REDUCE/STIMULATE
IL-8	25	0.0038	↓	IL-12p70	26	0.022	↓
IL-2	24	0.0269	↓	VEGF-A	24	0.0229	↓
IL-13	25	0.0342	↓	IL-8	25	0.0255	↓
VEGF-A	24	0.0366	↓	VEGF-C	20	0.0296	↓
TNF-α	25	0.0451	↓	IL-13	25	0.0342	↓
IL-12p70	26	0.0463	↓	TNF-α	25	0.0393	↓
VEGF-C	20	0.0696	↓	PIGF	24	0.0395	↓
IL-4	25	0.0903	↓	IL-2	24	0.0491	↓
INF-y	24	0.2479	↓	INF-y	24	0.0691	↓
VEGF-D	12	0.2633	↑	IL-4	25	0.1014	↓
PIGF	24	0.4223	↓	IL-10	25	0.2635	↓
IL-6	25	0.5782	↓	IL-6	25	0.3254	↓
IL-10	25	0.7112	↓	FLT-1	23	0.4274	↓
bFGF	23	0.754	↓	TIE-2	22	0.5446	↓
TIE-2	22	0.799	↓	VEGF-D	12	0.5879	↓
FLT-1	23	0.7998	↓	IL-1β	25	0.8325	↓
IL-1β	25	0.8119	↓	bFGF	23	0.9168	↓

Interleukin(IL)-1β, 2, 4, 6, 8, 10, 12p70, 13, tumor necrosis factor(TNF)-α, interferon(IFN)-γ from colonic explants. Secreted levels of angiogenic proteins basic fibroblast growth factor (bFGF), vascular endothelial growth factor receptor 1 (Flt-1), placental growth factor (PIGF), angiopoietin receptor (Tie-2) and vascular endothelial growth factor (VEGF) A, C and D

IFN-γ

IL-10

IL-12p70

IL-13

IL-1β

IL-2

IL-4

IL-6
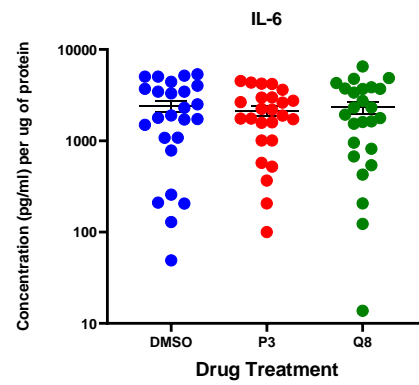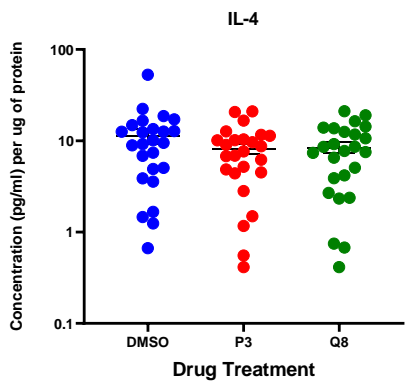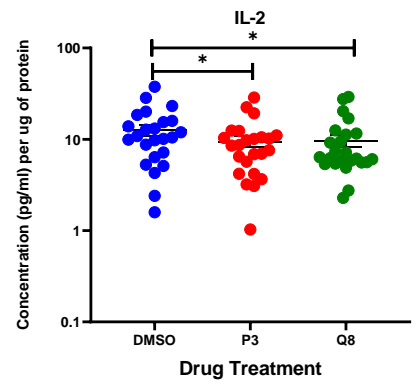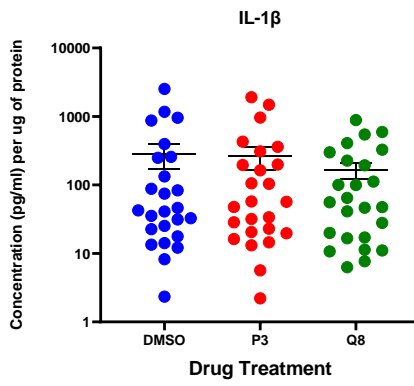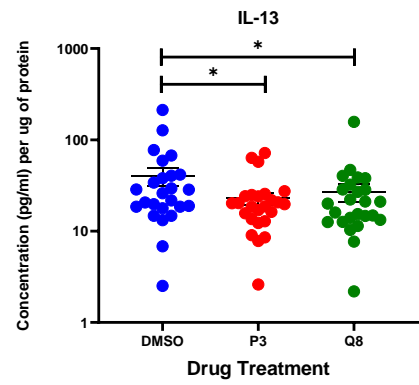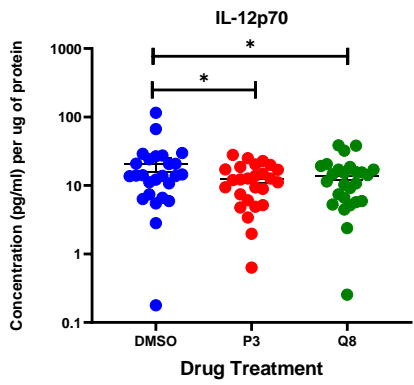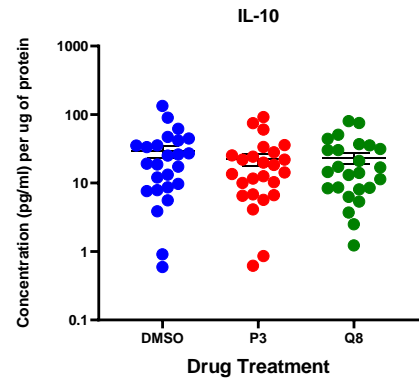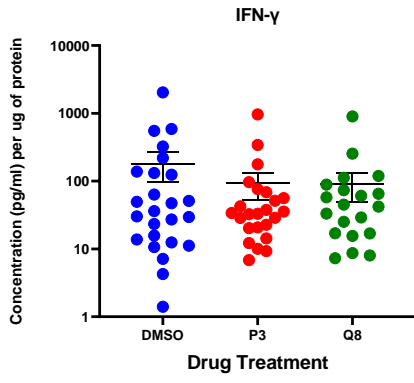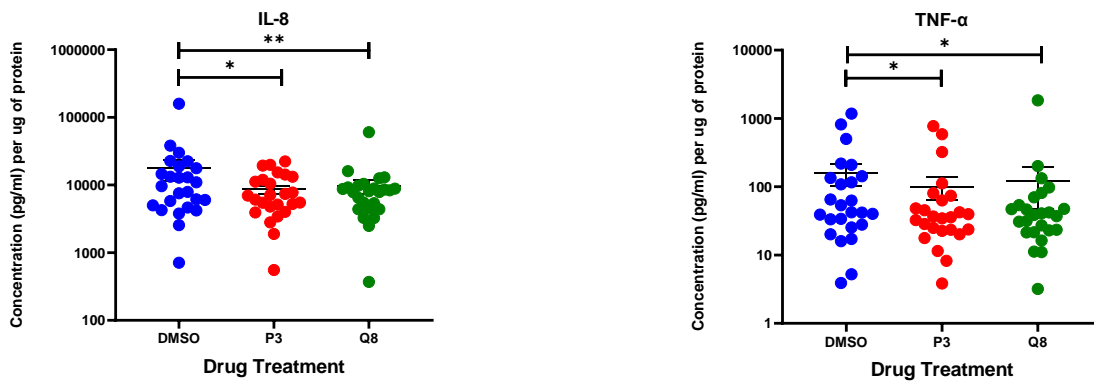
Figure 4.1 *Inflammatory analyte expression following treatment with control (DMSO), P3 and Q8.*

Both drugs cause significant reduction in TNF-α, IL-2, IL-8, IL-12p70 and IL-13. Wilcoxon signed rank test. Statistically significant if P value <0.05, denoted with *, <0.001, denoted with **. Line denotes mean with standard error of mean (SEM).
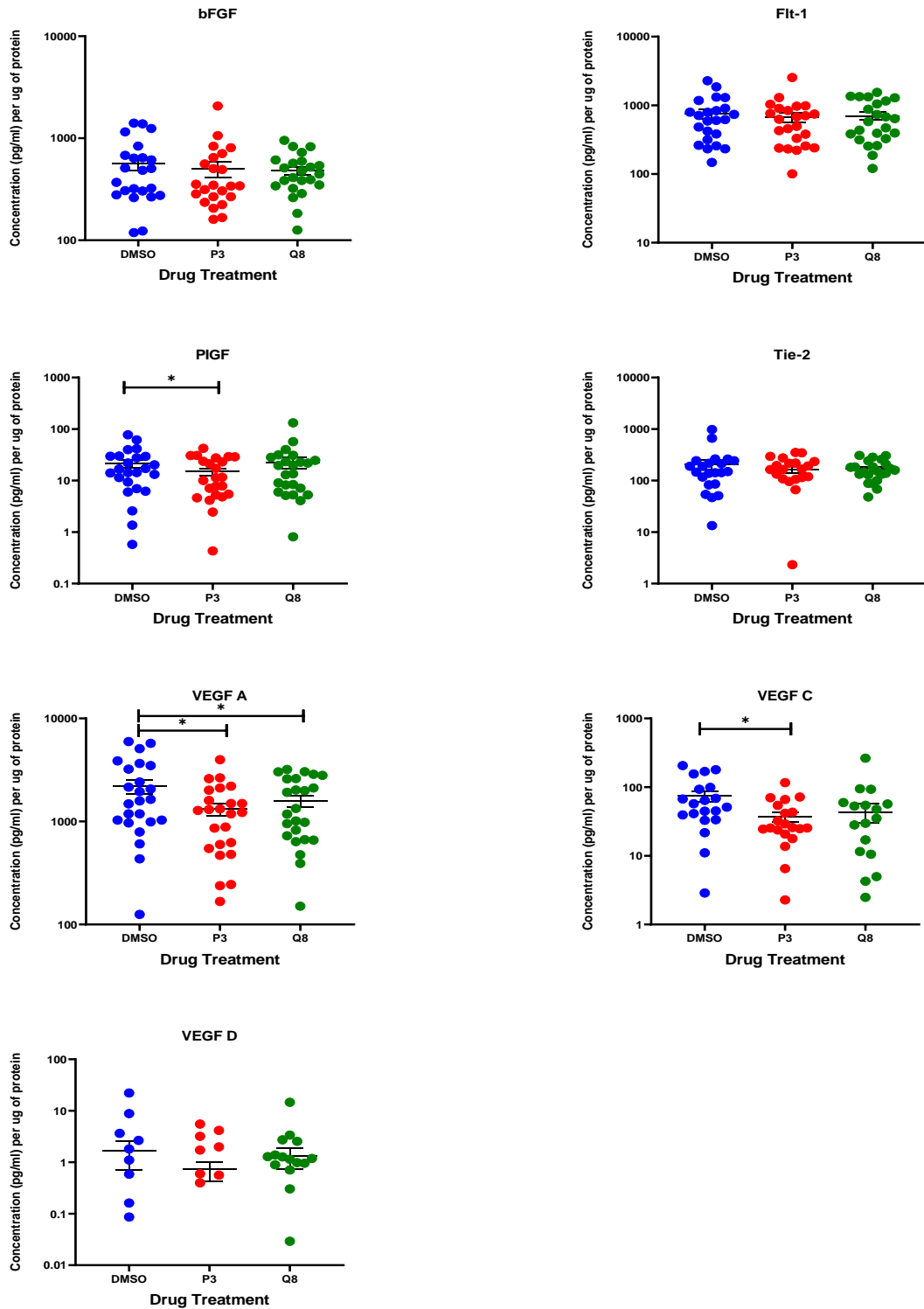
Figure 4.2 *Angiogenic analyte expression following treatment with control (DMSO), P3 and Q8.*

Both drugs cause significant reduction in VEGF-A. In addition, P3 causes downregulation of VEGF-C and PIGF. Wilcoxon signed rank test. Statistically significant if P value <0.05, denoted with *. Line denotes mean with standard error of mean (SEM).

4.4.2 Effect of P3 and Q8 on analyte expression in biologic exposed and naïve cohorts

Biologic exposure is a key factor in therapy response in UC. The biologic status of patients was evaluated and the effects of P3/Q8 were investigated in these subgroups. Patients with prior biologic (9 patients) exposure or on current treatment were included in the biologic exposed group. Patients who had never received a biologic agent were classified as biologic naïve (17 patients). All patients in the biologic exposed group had at one time received an anti-TNF treatment. Patients in the biologic exposed group were on a variety of different agents including: infliximab, adalimumab, golimumab, ustekinumab and vedolizumab. Analyte expression for each of treatment type within these groups was measured and compared with control. In the biologic exposed group P3 caused a significant reduction in inflammatory analytes IFN- γ, IL-4, IL-12p70, IL-13 and TNF-α. The reduction in TNF-α occurred despite most of the patients being on an anti-TNF agent. Q8 did not elicit a significant reduction in any inflammatory analyte in the biologic exposed group. P3 also reduced VEGF-A expression in the biologic exposed group when compared with control.

P3 did not display any anti-inflammatory or anti-angiogenic activity in the biologic naïve subgroup. Q8 downregulated IL-8 significantly but did not exert any effect on other inflammatory analytes. Q8 also reduced VEGF-A and VEGF-D expression.
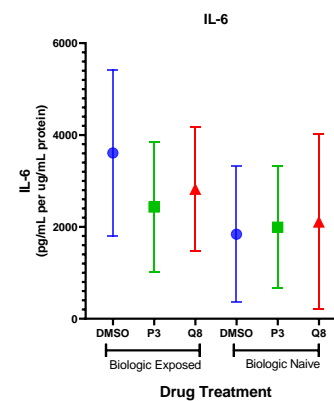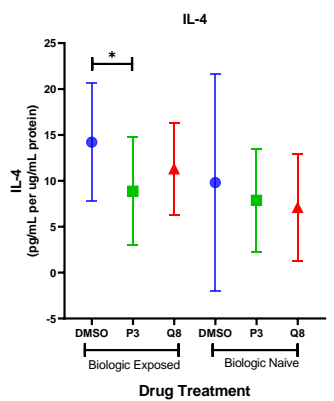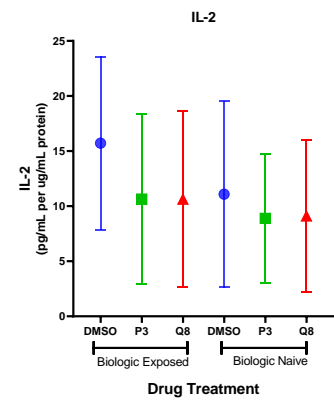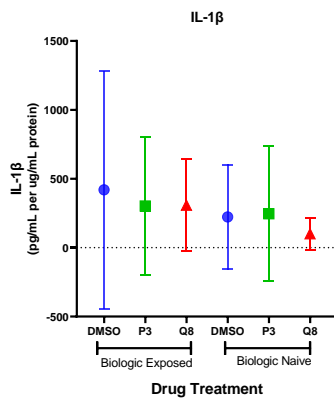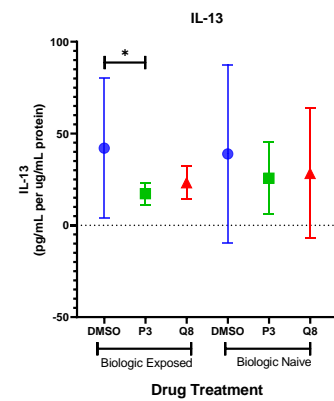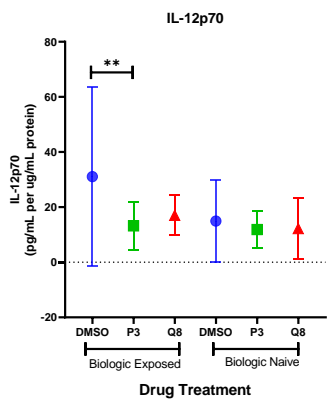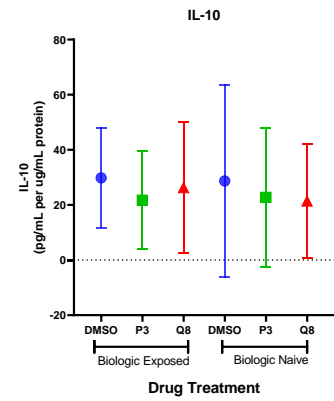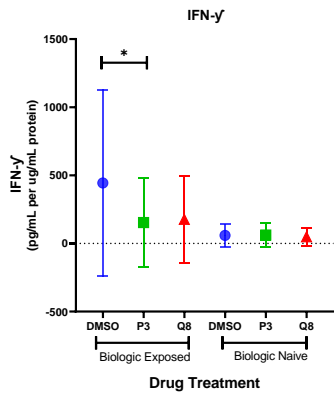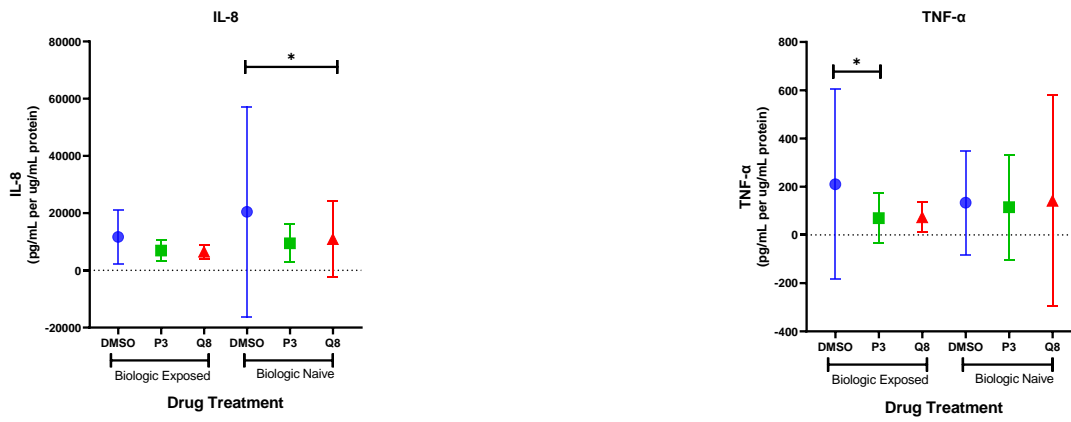
Figure 4.3 *Inflammatory analyte expression following treatment with control (DMSO), P3 and Q8 in biologic exposed and naïve subgroups.*
Wilcoxon signed rank test. Statistically significant if P value <0.05, denoted with *, <0.001, denoted with **.
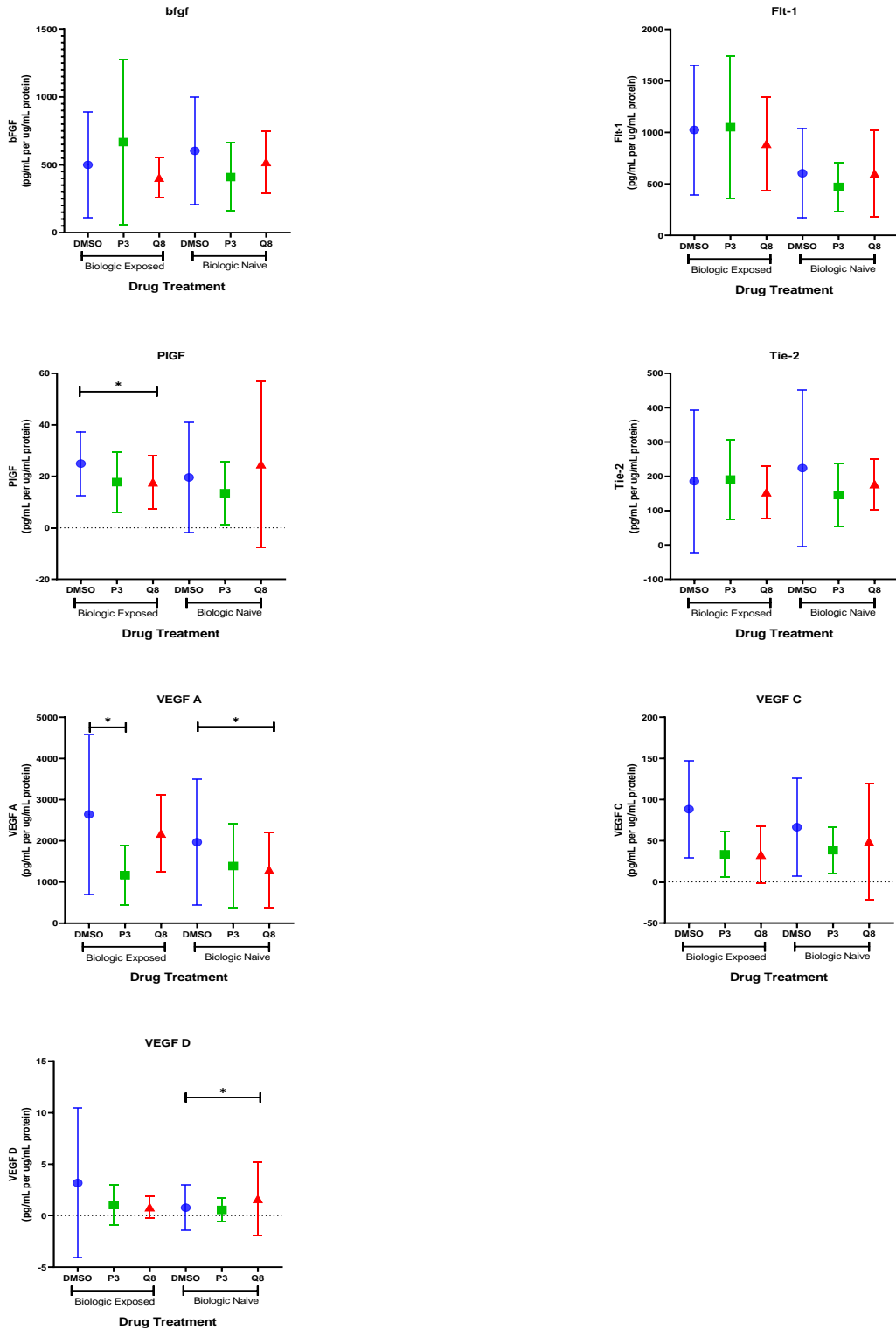
Figure 4.4 *Angiogenic analyte expression following treatment with control (DMSO), P3 and Q8 in biologic exposed and naïve subgroups.*
Wilcoxon signed rank test. Statistically significant if P value <0.05, denoted with *.

4.4.3 Effects of P3 and Q8 on analyte expression in high vs normal CRP cohorts

CRP is an accurate marker of inflammation. In this study we have shown that an elevated CRP is associated with increased disease severity. Partial, endoscopy and total Mayo score correlated positively with an elevated CRP. The presence of bleeding at colonoscopy was also associated with increased CRP. Patients were grouped according to their CRP levels with an increased CRP defined as >5mg/L and a normal CRP <5mg/L. The treatment effect of both P3 and Q8 was compared with control to evaluate whether either drug was more effective in elevated or normal CRP patients.

In the cohort with CRP less than 5mg/L, inflammatory marker IL-8 was suppressed by Q8, none of the other inflammatory analytes were significantly affected. P3 did inhibit secretion of Tie-2 in this cohort.

In the group with elevated CRP (>5mg/L) VEGF-A was inhibited by P3, while administration of Q8 resulted in suppression of PIGF and VEGF-C. Neither drug caused significant reduction in any of the inflammatory analytes. Analytes in which a significant therapeutic effect was elicited are included in Figure 4.5, graphs with all analytes are included in the appendix.
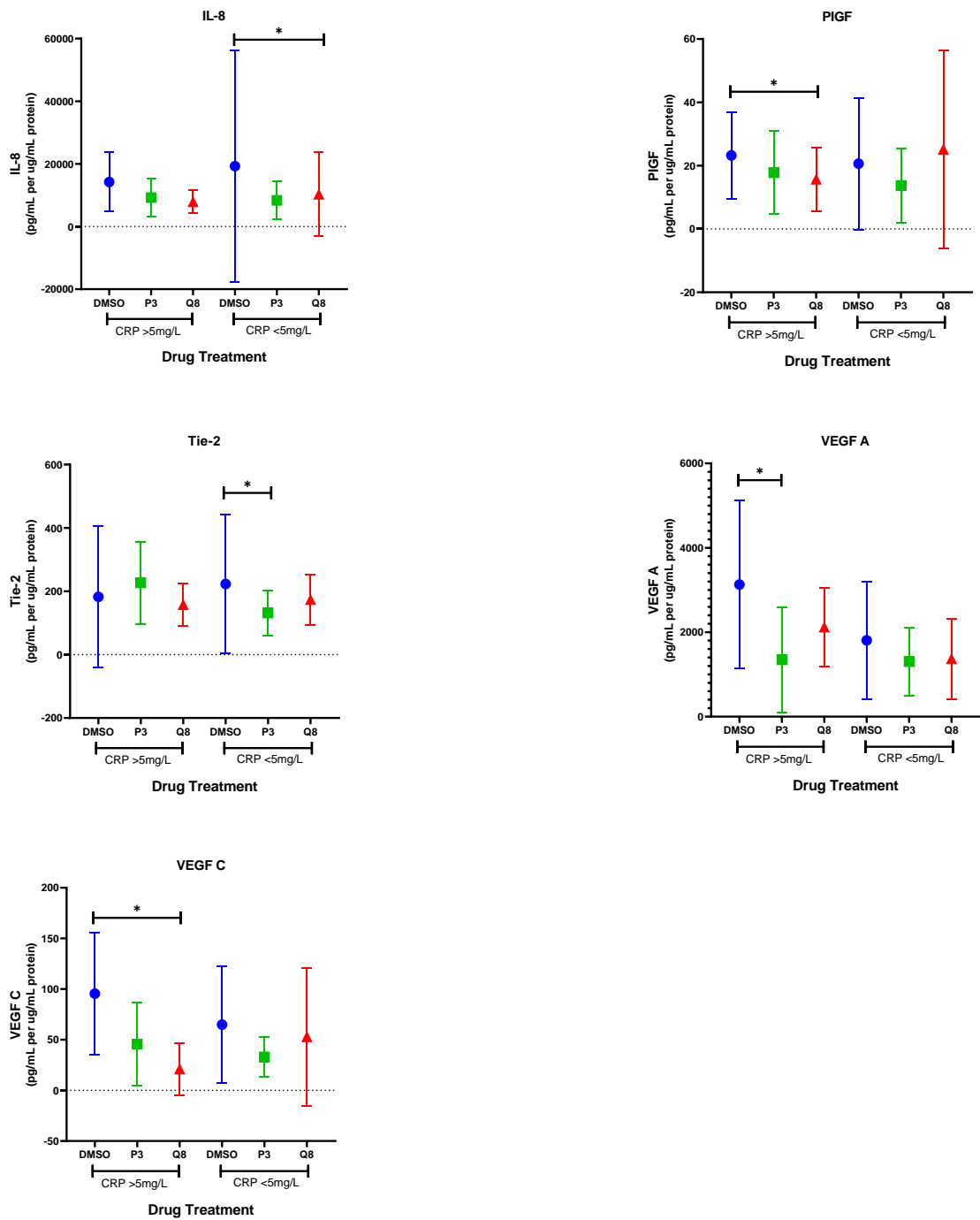
Figure 4.5 *Inflammatory and angiogenic analyte expression following treatment with control (DMSO), P3 and Q8 in patients with CRP <5mg/L and CRP >5mg/L.*
Only analytes were significant change occurred are included. Wilcoxon signed rank test. Statistically significant if P value <0.05, denoted with *.

4.4.4 Effect of P3 and Q8 on analyte expression in longstanding UC

P3 and Q8 were evaluated in subgroups with UC >10 years and <10 years. Amongst patients with UC for greater than 10 years P3 displayed anti angiogenic effects by inhibiting expression of TIE-2, VEGF-C and PlGF. In this patient group neither agent significantly inhibited any of the inflammatory analytes. VEGF-D expression was significantly increased in colonic samples treated with Q8 when compared with DMSO.

In the cohort with UC for less than 10 years both P3 and Q8 significantly inhibited the expression of key inflammatory analytes IL-8, IL-12p70 and TNF-α. Both agents also caused a reduction in VEGF-A and Q8 suppressed the secretion of VEGF-C. Analytes in which a significant therapeutic effect was elicited are included in Figure 4.6, graphs with all analytes in the appendix.
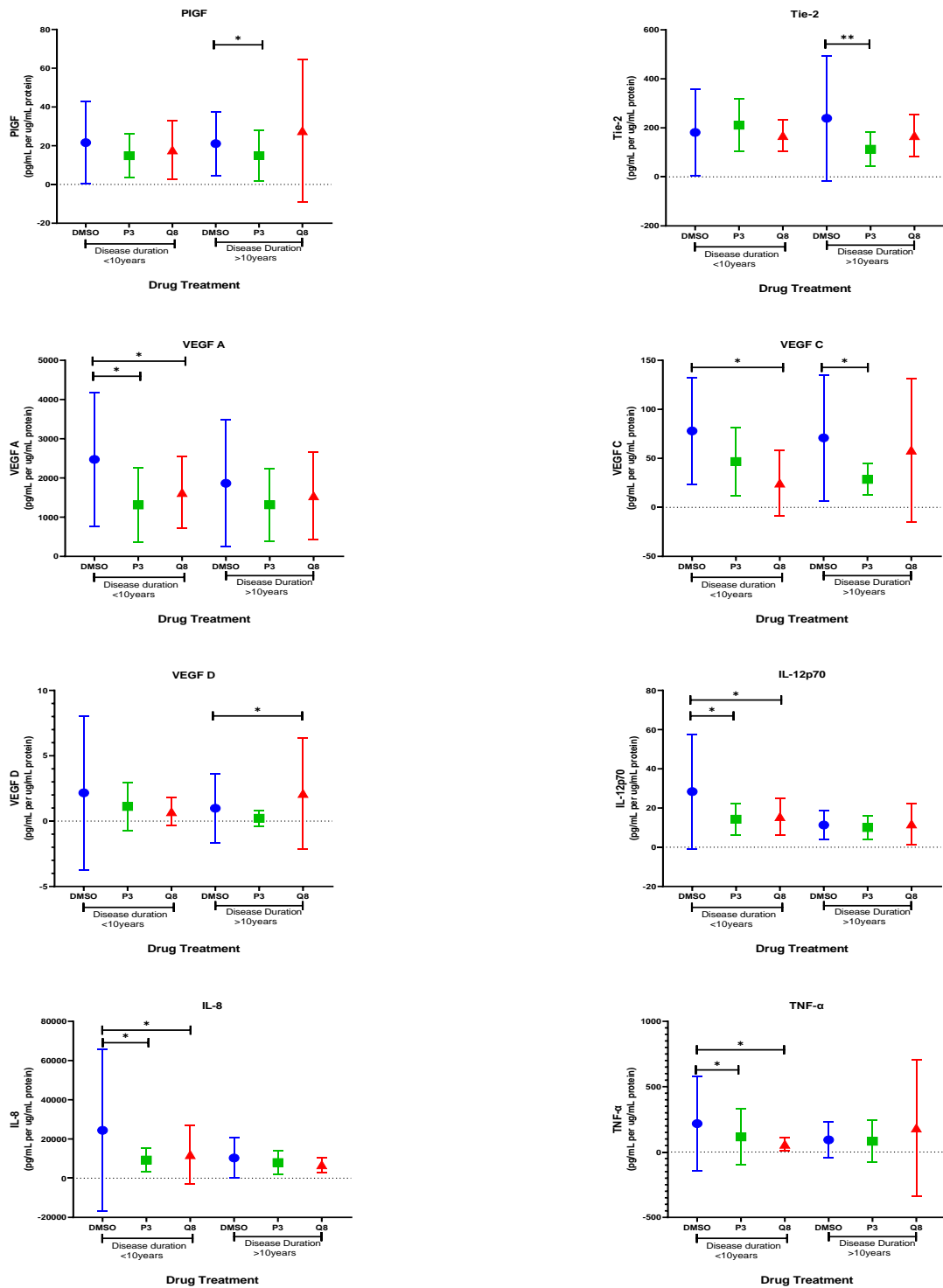
Figure 4.6 *Inflammatory and angiogenic analyte expression following treatment with control (DMSO), P3 and Q8 in patients with UC for >10 years and <10years.* Only analytes were significant change occurred are included. Wilcoxon signed rank test. Statistically significant if P value <0.05, denoted with *.

4.4.5 Effects of P3 and Q8 on analyte expression in UC samples with inflammation on histopathology

Analyte expression in patients with inflamed and non-inflamed histopathology was analysed. Treatment with P3 led to a significant reduction in VEGF-A secretion in the inflamed cohort. Q8 resulted in a reduction in IL-8 expression in the cohort with non-inflamed histology (Figure 4.7). No other analyte was significantly affected.
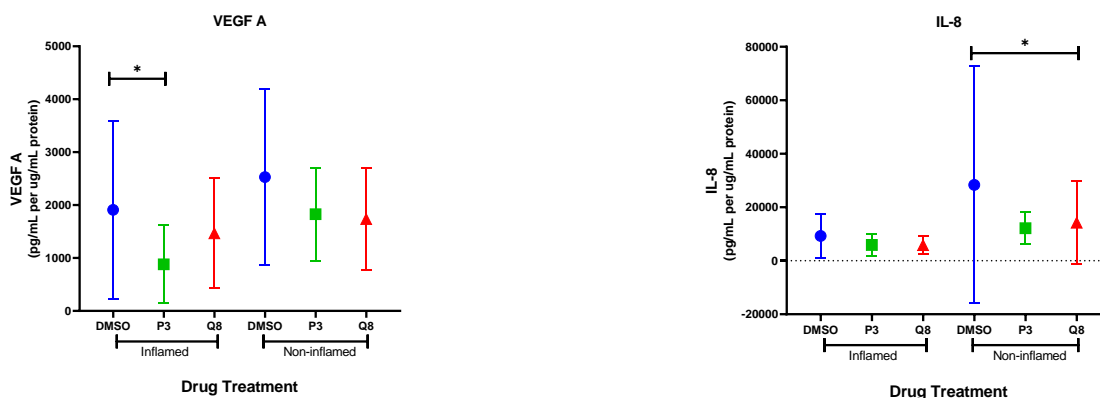
Figure 4.7 *Inflammatory and angiogenic analyte expression following treatment with control (DMSO), P3 and Q8 in patients with inflamed and non-inflamed histopathology on colonic biopsies.*
Only analytes were significant change occurred are included. Wilcoxon signed rank test. Statistically significant if P value <0.05, denoted with *.

4.4.6 Comparing efficacy of P3 and Q8 at reducing inflammatory and angiogenic analyte expression

To compare the performance of both small molecule agents the Log2 fold change between treatment and control was calculated for each analyte. This was mapped using heatmaps in r studio and analysis was carried out using Wilcoxon signed rank test on graphpad. The secretion of analytes were reduced in the majority of cases when treated with Q8 compared with control. In some cases, there was a mixed response with some analytes reduced and some increased. One of the patients was an outlier, for patient 18 all inflammatory and angiogenic analytes were significantly up regulated, the reason for this is unclear (Figure 4.8). Similarly, with P3 the majority of analytes were suppressed when compared with control, but some were upregulated. The inhibition of inflammatory analytes appears more profound than that of angiogenic analytes (Figure 4.9).

An analysis was performed to compare the efficacy of each agent and determine if one outperformed the other. P3 caused a statistically significant reduction in PIGF compared with Q8. Changes between the log2 fold change in all other analytes were none significant (Figure 4.10 and 4.11).

Q8 post treatment log2 fold change

Angiogenic Analytes (above)

Patient	2	3	4	5	6	7	8	9	10	11	12	13	16	17	18	19	20	21	22	23	24	25	26
bFGF	-0.2		-1.8	-0.1	0.8	1.2	0.9	0.9	0.5	-0.4	0.1	1.5	1.7	-1.2	0.6	-0.2	-0.1	-0.3	-2.9	-1.1	0.4	-0.4	-1.4
Flt-1		-0.5	0.6	0.8	0.6	-0.1	0.7	0.7	0.1	-0.3	-0.4	1.5	0.6	-1.4		-0.4	-0.7	-0.3	-1.6	-0.4	-0.3	0.4	-1.3
PlGF	-0.5	0.2	-0.8	0.6	-0.4	0.5	4.1	0.4	-1.0	-0.3	0.2	1.7	1.0	-3.5	3.4	0.5	-1.2	-1.4	-1.4	-0.6	-0.4	1.1	-1.1
Tie-2	4.2	-0.4	0.6	-0.1	-0.6		0.9		-2.9	-1.6	1.2	X	-1.1	-1.6	1.6	-0.1	-0.5	-1.0	1.4	0.4	2.5	0.3	-1.7
VEGF	-0.8	-0.6	-0.7	0.3	-1.4	-1.1	-0.2	1.0	0.4	0.4	-1.1	0.2	-0.3	-1.8	1.4	-0.6	-0.3	-0.2	0.1	-0.6		0.6	-1.6
VEGF-C	-1.5	-0.2	1.4	-0.1	-0.3	0.9	X	0.7	-4.0	-1.0	-0.3	X	0.4	X	2.6	X	-4.2	-3.0	X	X	-4.2	0.3	-2.6

Q8 post treatment log2 fold change

Inflammatory Analytes (below)

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	16	17	18	19	20	21	22	23	24	25	26
IFN-γ	1.5	-0.9	-1.0	-0.3	0.3	-1.7	-0.4		1.3	0.6	X	-1.0	-0.8	X	X	X	0.8	0.9	-2.2	0.6	-0.3	X	-0.3	0.7	-1.5
IL-10	1.5	-0.1	0.3	-1.6	1.0	0.4	0.5	1.3	-0.2	-0.1	0.4	-3.3	-0.1	-1.6	-1.2	-1.6	3.2	-0.6	-1.8	1.1	-1.3	-0.6	0.5	0.9	-1.0
IL-12p70	2.4	-0.5	-0.7	-0.9	0.5	-0.5	1.4		0.5	-0.5	0.4	0.1	-0.4	-2.0	-0.5	-3.5	1.7	-0.4	-1.4	-0.1	-1.0	-1.4	0.2	-0.4	-0.2
IL-13	0.7	-2.5	-1.2	-0.4	-0.2	-0.9	0.9	-0.9	0.2	0.3	-0.6	-0.6	0.6	-0.4	-0.5	-2.9	1.2	-0.6	-1.2	-0.6	0.5	-0.6	-0.3		-1.5
IL-1ß	2.1	2.0	-1.7	-1.1	-0.7	1.1	-3.5	1.8	0.3	1.0	0.6	-0.3	-0.4	-1.1	-0.7	-2.0	2.2	0.2	-1.1	-0.1	-1.5	-0.3	0.4	0.8	0.3
IL-2	0.1	-1.9	-1.2	0.1	X	-1.3	0.3	0.1	1.1	-0.1	-1.2	-0.8	-0.4	-0.4	-0.8	-1.2	2.0		-1.3	-0.2	-1.2	-0.6	1.2	0.2	-1.3
IL-4	2.1	-0.3	-0.3	-0.7	-0.7	0.1	0.7	1.3	0.6	-0.2	-0.3	-0.6	-0.9	-2.1	-2.9	-2.4	1.7	-0.2	-1.2	0.9	-1.5	-1.7	1.2	-0.4	-1.5
IL-6	2.1	0.2	-0.8		-1.8	-0.5	0.4	4.9	0.9	-0.1	-0.3	-0.6	1.0	-0.1	-2.7	-2.4	1.9	-1.1	-1.3	1.4	-1.8	-1.4	2.0	0.1	-1.3
IL-8	-0.6	-2.7	-0.8	0.3	-0.9	-0.5	1.4	-2.0	-0.1	-0.1	-0.4	-0.4	-0.2	-1.4	-0.3	-2.2	1.8	-0.5	-1.4	-0.6	0.3	-0.7		0.1	-1.1
TNF-a	0.7	-0.8	-2.3	-0.2	-0.3		-4.1	-0.8	0.2	0.6	0.2	-0.8		-1.6	-0.8	-2.5	2.0	-0.2	-1.9	-0.3	-2.6		0.4	1.9	-1.0

Figure 4.8 *Heatmaps of analyte Q8 vs control.*
These heatmaps display log2 fold change of analyte expression following treatment with Q8 compared to control. Angiogenic panel (above) and inflammatory panel (below). Darker colour indicating more significant inhibition of analyte.

Angiogenic Analytes — Patient

Analyte	2	3	4	5	6	7	8	9	10	11	12	13	14	16	17	18	19	20	21	22	23	24	25	26
bFGF	0.3	-1.2	-1.7	0.4	1.2	1.2	-0.2	0.6	-0.4	0.1	-1.2	0.9	X	1.0	-1.0	-0.2	0.8	0.3		0.6	-0.6	-0.7	-1.9	-2.4
Flt-1	-0.3	-1.3	0.8		0.5	0.4	-0.2	0.3	-0.2	-0.5	-1.2	1.1	X	0.2	-1.1	-0.1	0.4	0.4	0.1	0.2	-0.1	-0.5	-0.8	-1.8
PIGF	-0.5	-1.3	-0.9	-0.4	-0.4	-0.8	3.1	0.1	-2.5	-0.4	-2.2	0.9	-1.4	0.4	-1.2	X	1.0	-1.6	-1.4	1.2	0.3	-4.0	-0.5	-0.8
Tie-2	4.5	-1.3	0.3	-0.2	0.7	0.3	-0.4	-0.4	-2.6	-0.3	-0.3	X	X	-5.9	-0.3	X	1.2	1.0	-0.4	2.0	0.7	X	-0.9	-2.6
VEGF	-2.5	-1.8	-0.3	0.4	-2.1	-2.7	-0.1	0.7	-1.8	-0.2	-1.4	0.2	-1.2	0.1	-1.3	0.8		-1.2	0.6	-1.3	0.2	-0.2	0.1	-1.3
VEGF-C	-1.8	-1.2	1.2	-0.6		0.1	X	0.3	-2.5	-1.7	-1.5	X	X	-3.0	-2.0	3.1	1.8	-5.4	-0.7	X	0.7	-1.7	-0.4	-2.8

Colour scale: 0, -5

Inflammatory Analytes — Patient

Analyte	1	2	3	4	5	6	7	8	9	10	11	12	13	14	16	17	18	19	20	21	22	23	24	25	26
IFN-γ	3.5	-1.7	-0.4	-1.0	-0.5	-2.0	0.1	0.5	0.1	-2.9	-1.1	-1.1	-0.4	-2.0	-0.4	2.3	X	0.1	-3.9	0.4	-0.6	3.1	-0.6	-0.3	0.3
IL-10	2.8	-0.7	-0.1	-1.8	0.5		-1.5	1.1	1.2	-1.4	-0.3	-3.3	0.7	-2.7	-0.3	-0.2	-0.6	0.4	-2.1	1.6	-0.3	0.2	-0.9	-0.2	0.5
IL-12p70	1.3	-0.7	-1.4	-1.1	1.8	-0.7	-0.9	0.6	-0.3	-2.5		-0.2	-0.2	-2.0	1.0	-1.6	0.3		-2.2	2.1	-0.1	-0.6	-1.6	-0.1	0.5
IL-13	1.1	-2.4	-1.0	-0.1	0.1	-0.2	-0.1	-0.8	-0.8	-1.0	-0.6	-1.0	0.1	-1.9	-0.2	-2.2	1.2	0.3	-1.7	0.3	0.1	0.4	-1.3	0.5	-2.2
IL-1ß	6.4	0.3	-1.4	-2.2	-1.2	0.3	-0.3	0.6	0.6	0.8	0.1	-0.1	-0.3	-1.9	0.3	-0.9	-0.1	0.5	-1.4	0.7	-0.8	0.5	-0.5	0.3	1.2
IL-2	0.2	-1.4	-1.8	0.1	X	-0.6	-1.4		1.2	-0.8	-0.8	-0.7	0.4	-1.0		-0.3	-0.6	0.2	-1.5	0.6	-0.9		0.4		-0.9
IL-4	2.1	-0.8	-1.1	-1.4	-0.7	-0.4	-0.6	1.1	-0.4	-2.3	-0.4	-0.6	0.1	-2.2	-1.7	-0.5	-1.2		-1.4	2.6	0.2	0.5	-0.5	0.4	-0.4
IL-6	0.6	0.3	-1.1	-0.3	1.0	-0.8	-0.5	5.1	0.3	-2.3	-0.4	-0.5	1.2	0.7	-0.6	-0.9	-0.3	-0.8	-1.3	3.1	-0.4	-0.8	0.8	-0.2	0.6
IL-8	0.2	-2.1	-1.2	0.4	-0.4	0.3	-0.2	-1.5	0.5	-0.3	-0.6	-0.3	-0.5	-3.0	1.0	-1.8	0.9		-1.5	-0.1	-0.2	-0.1	-0.6	-0.3	-1.8
TNF-a	1.7	-2.0	-1.7	-1.2		-0.7	-0.1	-0.8	0.4	-0.5	-0.8	-1.0	0.1	-2.3	0.8	-1.6	0.7	0.5	-2.0	0.2	-1.9	0.6	-0.6	0.2	-0.8

Colour scale: 6, 4, 2, 0, -2

Figure 4.9 *Heatmaps of analyte P3 vs control.*
These heatmaps display log2 fold change of analyte expression following treatment with P3 compared to control. Angiogenic panel (above) and inflammatory panel (below). Darker colour indicating more significant inhibition of analyte.

Figure 4.10 *Comparison of Log2 fold change between P3 and Q8 for each inflammatory analyte.*

No significant differences noted. Only analytes were significant change occurred are included. Wilcoxon signed rank test. Statistically significant if P value <0.05, denoted with *.
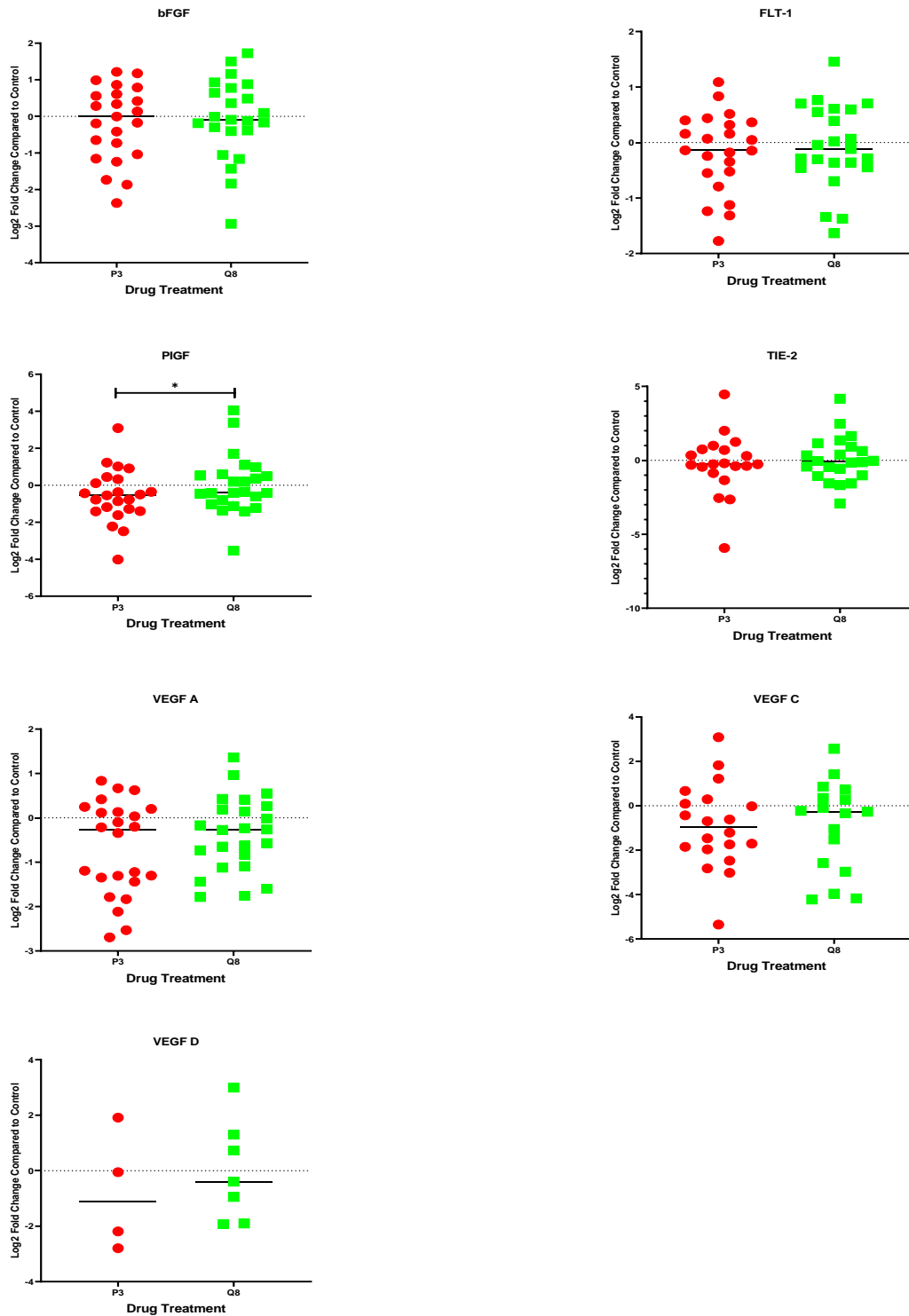
Figure 4.11 *Comparison of Log2 fold change between P3 and Q8 for each angiogenic analyte.*
P3 treatment resulted in significant PIGF inhibition compared with Q8. No other differences were noted. Wilcoxon signed rank test. Statistically significant if P value <0.05, denoted with *.

4.5 Discussion

4.5.1 P3 and Q8 inhibit key inflammatory analytes

This study has demonstrated that key inflammatory analyte secretion is inhibited when colonic explants are treated with P3 or Q8. A statistically significant suppression of TNF-α, IL-2, IL-8, IL-12p70 and IL-13 was noted with both P3 and Q8. This confirms the potent anti-inflammatory activity of these small molecule agents.

TNF-α is a key target in UC therapeutics. TNF-α levels have been shown to correlate accurately with disease activity (142). An influx of white cells, predominantly eosinophils, to the intestinal mucosa occurs in active UC. This results in tissue damage, inflammation and ulceration of the mucosa. TNF-α is an important factor in stimulating this influx (143). Anti-TNF therapies have been used in UC for nearly 20 years. It has revolutionised the management of severe active UC and provided a therapeutic option where surgery would have previously been required (144). TNF-α has been consistently shown to be a key molecule in UC pathogenesis. Both novel small molecule agents, P3 and Q8, exert a significant inhibitory effect on TNF-α compared with control.

IL-12 is an important T helper cytokine that has been shown to drive intestinal inflammation. More recently IL-12 has become a therapeutic target in the management of UC. Novel biologic agent ustekinumab targets IL-12 and IL-23 with impressive results. It targets the p40 subunit on these interleukins and has been

shown to have a significant impact at achieving and maintaining remission in patients with moderate to severe UC (145). IL-12 has been shown to be upregulated in active UC and is thought to have an important role in pathogenesis (146). Most of the data evaluates the role of the p40 subunit, however the p70 subunit has been shown to be increased in active Crohn's disease. One study also found that the p70 subunit was downregulated following IL-12p40 blockade, suggesting significant overlap (147). P3 and Q8 both inhibit the secretion of IL-12p70 in our colonic explant model.

The data for the role of IL-2, IL-8 and IL-13 in UC is less robust. IL-2 has been shown to be increased in active Crohn's disease, however this was not reproduced in UC (148). A pilot study evaluating the role of IL-2 inhibitor, daclizumab, in a small cohort of UC patients gave promising results. The treatment was well tolerated and resulted in clinical and endoscopic improvement (149). However a randomized double blinded study of daclizumab found that patients in the treatment group where no more likely to be in remission at 8 weeks than those in the placebo group (150). Regardless, the treatment was taken off the market because of a reported increased risk in encephalitis amongst patients with multiple sclerosis (for which it was initially approved). No further studies were conducted to evaluate its role in UC. IL-8 has a role in intestinal inflammation but has not been therapeutically targeted. IL-8 is significantly higher in patients with active UC, compared with patients with CD or healthy controls (151). IL-8 correlate significantly with disease activity and may have a role as a marker of severity (152). IL-13 is a cytokine released from activated T-lymphocytes. It is a key cytokine in airway inflammation in asthma and also has a role in gastrointestinal inflammation. IL-13 is increased in colonic biopsies from patients

with UC, readings were significantly higher than in healthy controls (153). Interestingly, a reduction in IL-13 following infliximab therapy was predictive of good clinical response (154). Both IL-8 and IL-13 have significant roles in inducing and perpetuating colonic inflammation in UC.

4.5.2 P3 and Q8 inhibit potent angiogenic molecule VEGF-A

VEGF-A is the main driver of angiogenesis, it also has a role in lymphangiogenesis and haemangiogenesis. Dysregulated release of VEGF-A can induce pathological angiogenesis that perpetuates inflammatory conditions (155). Increased expression of VEGF-A in colorectal cancer is associated with lymphatic and systemic metastases (156). Both P3 and Q8 significantly reduced the secretion of VEGF-A when compared with control. The role of VEGF-A in UC is unclear, there is evidence that it is overexpressed in active disease, supporting the hypothesis that it causes pathological angiogenesis. In a paediatric population with IBD, VEGF-A was found to be increased compared with healthy controls, increased levels correlated with more severe disease activity (62). Infliximab indirectly inhibits VEGF-A secretion, this likely reduces inflammation-driven angiogenesis and contributes towards the clinical effects of the treatment. However, it is clear that angiogenesis is important in gastrointestinal healing and repair.

Treatment with P3 also caused significant reduction in VEGF-C and PIGF. The primary role of VEGF-C is lymphangiogenesis, which is achieved by stimulating lymphatic endothelial cells. The role of VEGF-C has not been fully evaluated in UC, however data

does exist in murine models. In these studies the data is conflicting, in one study stimulation of VEGF-C expression caused a deterioration in intestinal inflammation (56). In a second study stimulation of VEGF-C was shown to ameliorate experimental colitis (157). At present there no data has been published evaluating the role of PIGF in UC.

Both small molecule agents significantly inhibit secretion of key angiogenic molecule VEGF-A. The mechanism by which this occurs is unclear. Neither agent is thought to directly target VEGF-A. Both molecules inhibit cysteinyl leukotriene receptors. Angiogenesis is important in maintaining colonic homeostasis, indiscriminate blockade of angiogenesis is unlikely to provide a therapeutic effect. This has been demonstrated in the side effects of current licensed anti-VEGF therapies. However, a blended anti-inflammatory and anti-angiogenic effect as has been demonstrated by our small molecule drugs may provide a novel channel for delivery of therapy.

4.5.3 P3 exerts anti-inflammatory effect in biologic exposed patients

Biologic status is important when considering therapy options. In general, patients who have failed biologic agents are more difficult to treat and achieve remission. This cohort of patients tend to have more severe disease. Managing biologic exposed patients is an ongoing challenge. In this study the effect of novel small molecule agents was evaluated on a biologic exposed and biologic naïve cohort. 9 patients were categorized as biologic exposed and 17 as biologic naïve. Interestingly treatment with

P3 was associated with a significant reduction in secretion of inflammatory analytes IFN- γ, IL-4, IL-12p70, IL-13 and TNF-α in the biologic exposed patient cohort. P3 also caused reduction in VEGF-A. These findings are significant as this novel therapeutic approach may provide a treatment option for difficult to manage cases of UC. P3 did not display any significant activity in the biologic naïve patient cohort.

4.5.4 P3 reduces angiogenic analytes in TCM from patients with longstanding UC

Disease duration is an important factor in UC and often determines the risk of progression to malignancy. In many cases the acute inflammatory burden can subside and issues with colonic scarring, strictures and cancer come to the fore. Conventional medical therapy for UC address the dysregulated inflammation, once chronic and fibrotic damage is done medical therapies are ineffective. While this is more commonly an issue for patients with CD it is also important in UC where the risk of cancer increases with disease duration. P3 caused a significant reduction in the expression of TIE-2, VEGF-C and PIGF in a patient population with UC for greater than 10 years. None of the inflammatory analytes were significantly reduced in the patient group with longstanding UC. In patients with UC for less than 10 years both P3 and Q8 displayed activity against IL-8, IL-12p70 and TNF-α.

4.5.5 P3 and Q8 display similar efficacy in UC explants

An analysis was carried out to compare both agents and determine a lead molecule. Although P3 caused a significant reduction in PIGF when compared with Q8, no other

significant differences were found. Both agents display similar efficacy in the reduction of inflammatory and angiogenic analytes in UC.

4.6 Conclusion

P3 and Q8 display potent anti-angiogenic and anti-inflammatory effects in UC colonic explants. Both P3 and Q8 led to a significant reduction in VEGF-A. VEGF-A is the most potent driver of angiogenesis and has been therapeutically targeted successfully by a number of approved medications. Furthermore VEGF-A is associated with increased disease activity in IBD (62). P3 also inhibited VEGF-C and PIGF when compared with control. Both P3 and Q8 have a significant anti-inflammatory effect. Both agents suppressed the secretion of TNF-α, IL-2, IL-8, IL-12p70 and IL-13. TNF-α is an important therapeutic target in UC.

The management of patients with prior biologic exposure is complex. Patients that fail biologics tend to have more severe disease and achieving remission is more difficult. P3 displayed a significant anti-inflammatory effect in the biologic exposed patient group. P3 suppressed the release of IFN- γ, IL-4, IL-12p70, IL-13 and TNF-α when compared with control. P3 did not exert a significant effect on the inflammatory microenvironment in patients who were biologic naïve. This suggests that P3 may have a role in this difficult to treat subgroup of patients with UC. P3 displayed an anti-angiogenic effect in the subgroup of patients with longstanding UC. P3 caused inhibition of TIE-2, VEGF-C and PIGF. To date the target of medical therapies in UC

have been to reduce inflammation. This improves symptoms and reduces the risk of complications. However, targeting angiogenesis may provide protection against the development and spread of CRC. When both P3 and Q8 were compared in a head to head analysis they displayed similar efficacy. Neither agent significantly outperformed the other, although P3 did exert a reduction in PIGF when compared with Q8.

This research provides encouraging data to support a potential role for P3 and Q8 in the management of UC. Both agents display potent anti-inflammatory and anti-angiogenic effects. P3 appears to reduce inflammatory analyte excretion in the difficult to treat biologic exposed subgroup.

Chapter 5

Characterising the impact of P3 and Q8 treated UC explants on Dendritic Cell maturation and adaptive immunity

5.1 Introduction

In this chapter the effects of tissue cultured media (TCM) and novel small molecule drugs on dendritic cell (DC) maturation and adaptive immunity will be evaluated. DCs are antigen presenting cells that activate naïve T cells in response to stimulatory markers. DCs are present in most tissues in an immature state, they identify and capture antigens and undergo maturation in response to inflammatory mediators (158). As they mature they gain the ability to present antigens to T cells. DCs in the periphery express lymphocyte stimulatory molecules, move to lymphoid organs and release cytokines to trigger immune responses. DCs activate and tolerize T-cells to innate antigens, thereby preventing autoimmune reactions (159). Expression of markers such as CD80, CD83, CD86, CD54 and HLA-DR on DC surface is associated with maturation. In the past little was known about the function of DCs, with advances in technology these cells can be isolated from peripheral blood samples to allow biological and molecular analysis. DCs have been shown to be key cells in controlling the adaptive immune system response. In this chapter, human immature DCs will be generated from monocytes. DC maturation will be stimulated by adding Escherichia coli Lipopolysaccharide (LPS). ELISAs will be carried out on supernatants to measure IL-10 and IL-12p70 secretion. Cells will be stained and analysed via flow cytometry to assess markers of maturation. This will give an insight into the effects of our novel drugs on DC function and maturation.

5.2 Aims

1. Evaluate effect of TCM (DMSO/P3/Q8) on IL-10 or IL-12p70 secretion in DCs.

2. Evaluate whether TCM (DMSO/P3/Q8) alters DC maturation

3. Uncover differences between the effects of DMSO, P3 and Q8 on DC maturation.

5.3 Role of DCs in UC

Research has been carried out evaluating the role of DCs in UC. DCs are key in

perpetuation of the chronic inflammatory response that it characteristic of UC. Current

theories on UC pathogenesis point towards dysfunction of the colonic mucosal

immune system. This occurs in genetically susceptible hosts with environmental

factors and the gut microbiome playing a role. DCs are important in the mucosal

immune system and act as a bridge between innate and adaptive immunity (160). In

UC, DCs are activated, upregulated and release more pathologically relevant cytokines

(161). DCs derived from peripheral blood samples in a UC cohort showed increased

stimulatory capacity. The number of activated DCs in the UC group was significantly

higher when compared with control. Activated and mature DCs expressing CD83

antigen were also detected in inflamed colonic mucosa of patients with UC and CD

(162). DCs may have a role in the immune response seen in UC flares, there is also

evidence that they may play a role in CRC development (163). To date no studies have

successfully targeted DCs to evaluate a therapeutic effect, although, there is evidence

that depletion of DCs during lymphocytapheresis may inhibit the release of

inflammatory cytokines in UC (164). Another study showed that probiotic VSL3 and corticosteroids induced a favourable DC phenotype and resulted in increased regulatory cytokines and a reduction in inflammatory molecules (165). Potential therapeutic targets have been identified. The blockade of DC induced T-cell stimulation was suggested as a method of decreasing IL-23/IL-17 inflammatory cascade. However clinical trials are required to further investigate this potential therapeutic approach (166).

The role of IL-10 in UC pathogenesis is controversial. Although evidence exists to point towards a pro-inflammatory effect, the majority of the evidence suggests that IL-10 has a more complex role and may modulate inflammation. IL-10 is secreted by DCs and in this setting displays important anti-inflammatory effects on T-cells. The role of IL-10 in UC pathogenesis was first identified when IL-10 deficient mice were noted to develop spontaneous severe colitis (167). These findings have been supported by studies in humans showing an association between early onset UC and IL-10 deficiency (168). Although rare, patients with IL-10 deficiency or loss of IL-10 function often present with severe colitis usually in the first 3 months (169). Despite robust evidence pointing towards a role for IL-10 in UC pathogenesis, there has been limited success targeting IL-10 for therapeutic gain (170).

Inflammatory molecules in a neoplastic microenvironment promote angiogenesis and influence immune response by disabling DCs (171). Malignant tissue significantly impacts DC maturation. Tumour conditioned media from CRC samples was delivered

as a pre-treatment to monocyte derived DCs, upregulation of CD54, CD83, CD86 and HLA-DR was inhibited in response to LPS. IL-10 expression was enhanced while IL-12p70 secretion was reduced (158). A study comparing the effects of tumour cultured media from various GI cancer sites (oesophageal, colonic and rectal) found that although the effect on DC maturation differed, significant inhibition of DC TNF-α was consistently noted in each group (172). In this study oesophageal tumour cultured media appeared to be less inhibitory of DC maturation than colonic or rectal cancers. Interestingly, DC inhibition correlates positively with survival in patients with CRC on bevacizumab therapy. The inhibition of LPS-induced expression of CD83 and CD1d by tumor cultured media and the inhibition of IL-12p70, CD83 and CD1d by bevacizumab cultured media correlated significantly with survival. This demonstrates that inhibition of DC maturation not only exerts an effect on immune response but also influences the clinical outcome (173).

5.4 Results

5.4.1 LPS induce DC maturation

DCs were harvested from peripheral blood mononuclear cells (PBMC), all materials and methods are described in chapter 2. Treatment of DCs with LPS induced maturation. Markers of maturation CD11, CD80, CD83, CD86, CD40, HLA-DR and programmed death ligand-1 (PDL-1) were significantly upregulated following treatment with LPS. CD54 was non-significantly upregulated.
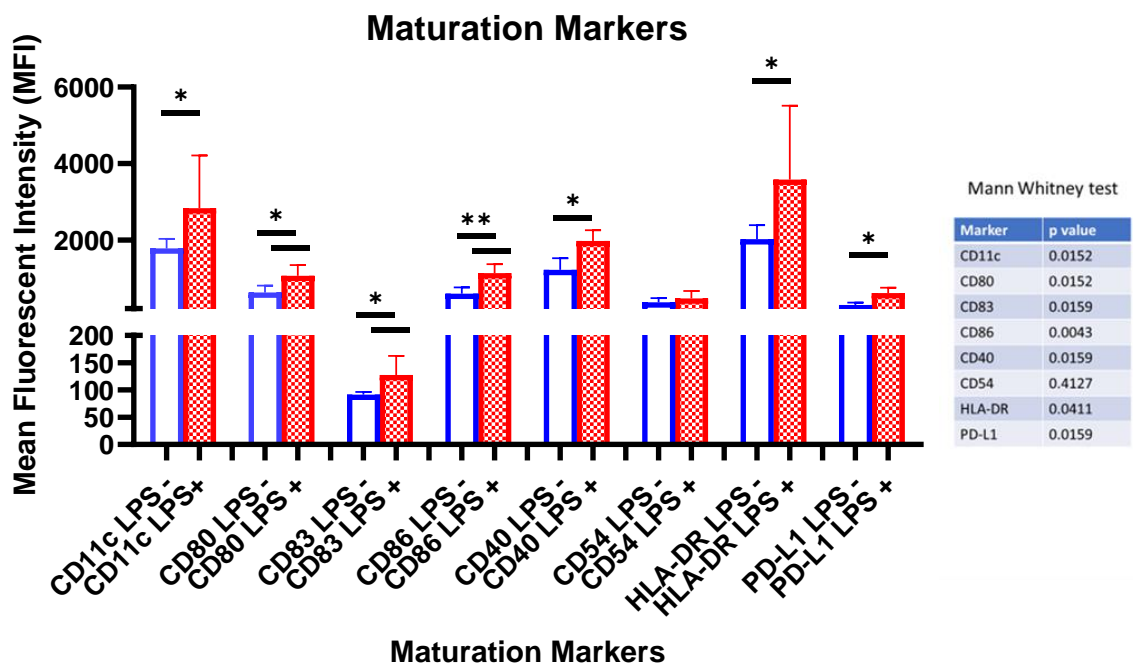
Figure 5.1 *DC maturation markers in LPS positive and negative settings.*
All markers displayed significant increase in response to LPS, with exception of CD54.

5.4.2 TCM alters DC maturation and IL-10 secretion

DCs were generated from PBMCs and treated with media only (M199) or TCM (DMSO/P3/Q8). Results for LPS negative and positive settings were obtained. TCM significantly altered markers of maturity and IL-10 secretion when compared with media only. IL-10 was significantly upregulated in the TCM groups. IL-12p70 secretion was similar in media only and TCM groups in LPS positive setting.
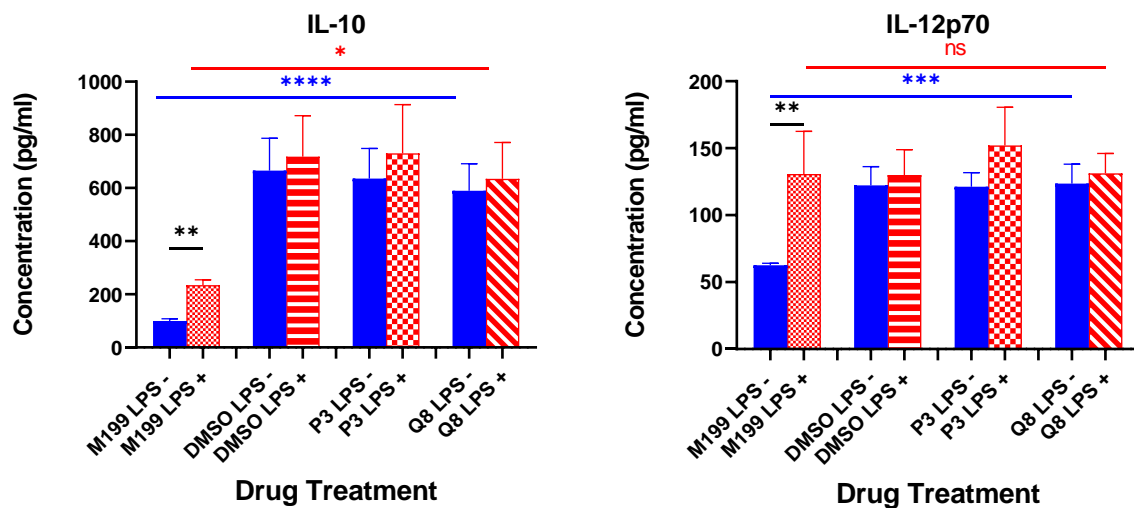
Figure 5.2 : *Dendritic Cell supernatant ELISA IL-10 and IL-12p70.*

IL-10 secretion was significantly upregulated in the TCM groups compared with M199 only.

5.4.3 P3 treatment results in CD54 and PD-L1 increase compared with DMSO

CD54 and PD-L1 expression was significantly higher in the DCs treated with P3 (Figure 5.3). No other significant differences were noted between markers of maturity in the DMSO, P3 and Q8 groups.
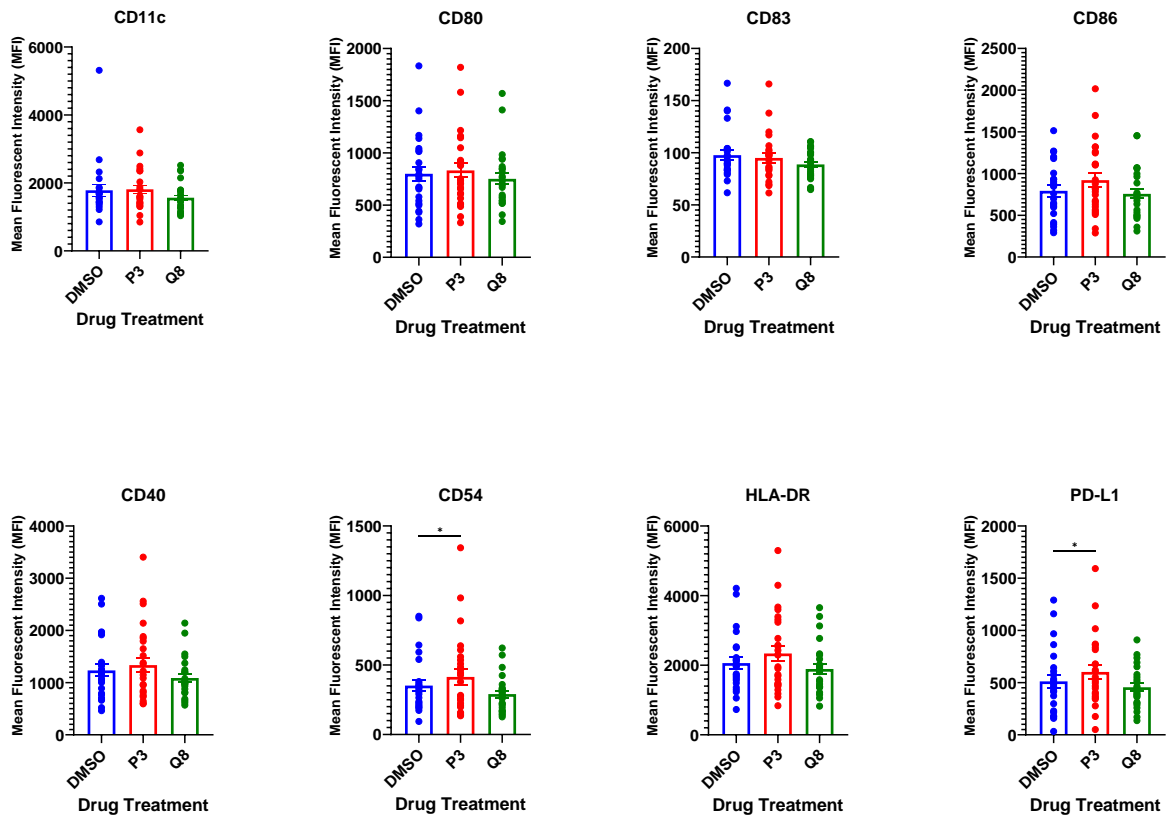
Figure 5.3 *Maturation marker expression in TCM treated with DMSO, P3 or Q8.*

Wilcoxon signed rank test. Clinically significant is P value <0.05.

5.4.4 TCM inhibits DC maturation in LPS positive setting

Markers of DC maturation were consistently highest in those DCs treated with media only LPS positive. All TCM groups displayed significantly lower levels of maturation markers CD11, CD80, CD83, CD86, CD40, HLA-DR and PDL-1. CD54 was also higher in the media only samples but the difference was non-significant.
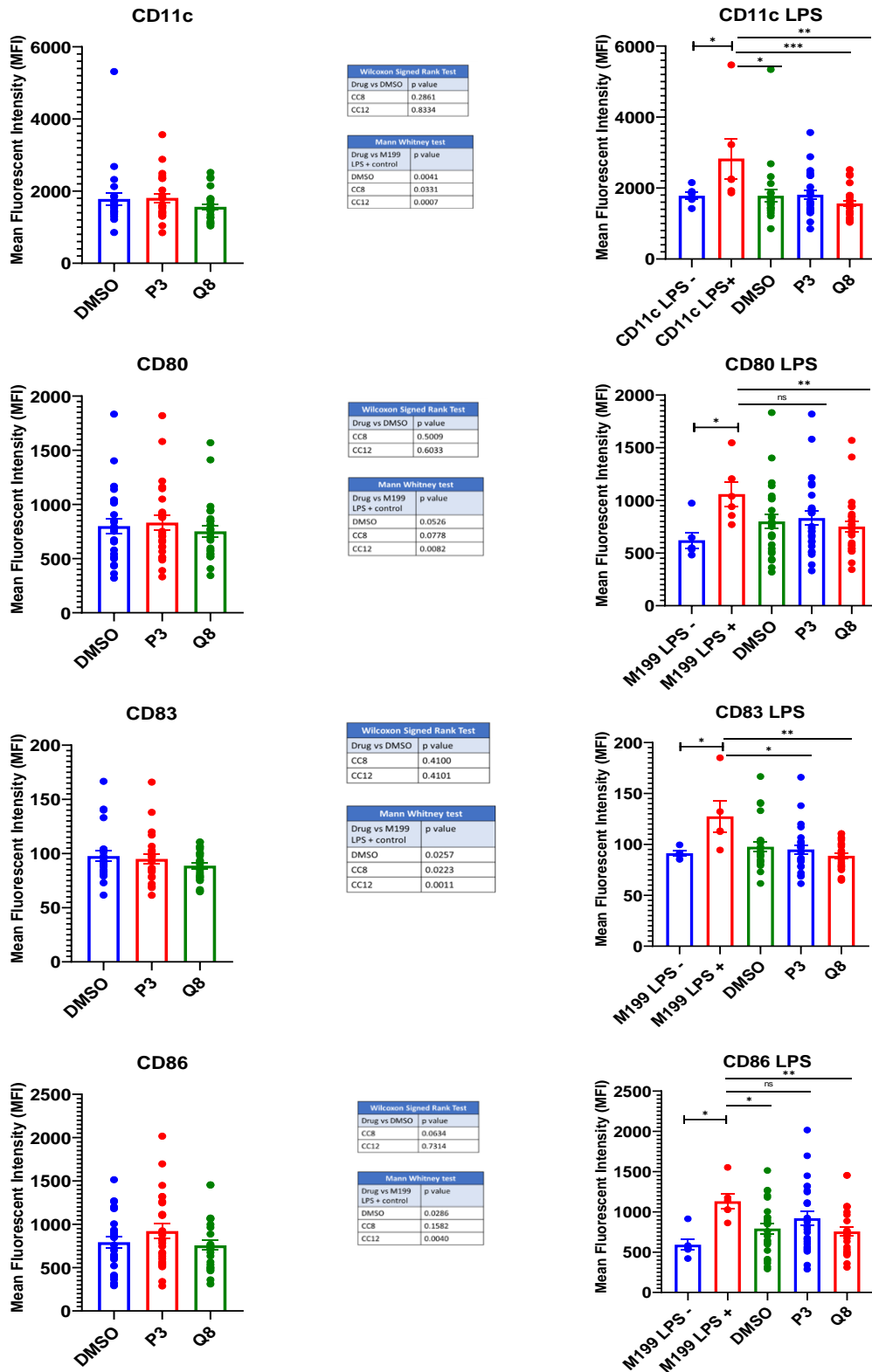
Figure 5.4 *Maturation marker expression in DMSO, P3 and Q8.*

On the left Wilcoxon signed rank test comparing P3 and Q8 to DMSO. On the right Mann Whitney test performed comparing LPS positive and negative settings. P values <0.05 significant, denoted with *, P values <0.01 denoted with **.
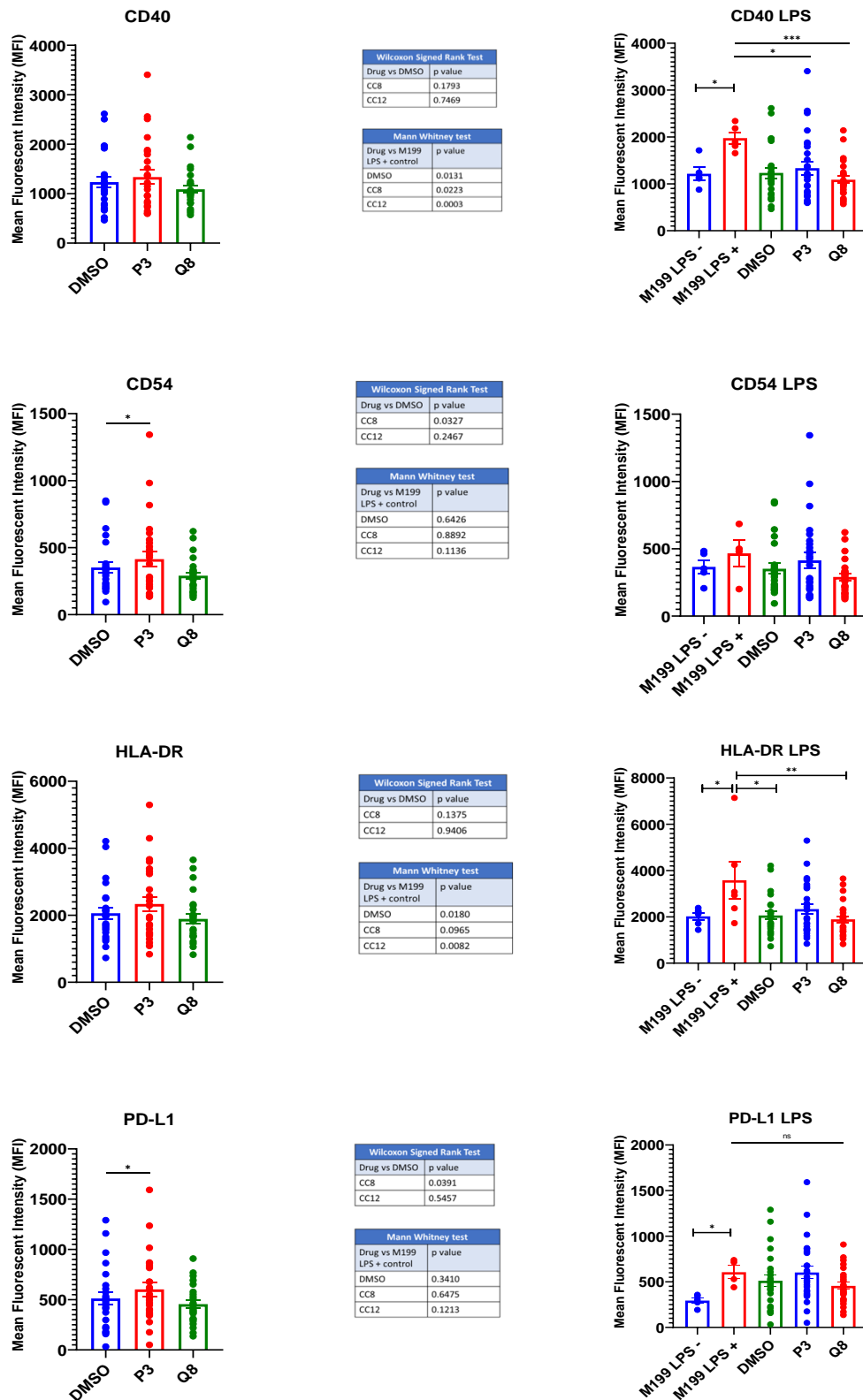
Figure 5.5 *Maturation marker expression in DMSO, P3 and Q8.*

On the left Wilcoxon signed rank test comparing P3 and Q8 to DMSO. On the right Mann Whitney test performed comparing LPS positive and negative settings. P values. P values <0.05 significant, denoted with *, P values <0.01 denoted with **.

5.5 Conclusion

5.5.1 TCM inhibits DC maturation in LPS positive setting

When compared with DCs treated with media only, TCM (DMSO/P3/Q8) was shown to exert a suppressive effect on DC maturation markers. All markers of maturation were significantly reduced compared with media only, with the exception of CD54. It is unclear what factors drive this difference in maturation. With the exception of a few molecules the findings between each TCM group were largely similar suggesting factors in the colonic microenvironment may cause suppression of the maturation markers. The role of DCs in UC is unclear, however emerging evidence suggests that they are important in disease pathogenesis. DCs are central players in the mucosal immune system and have a key role in regulating the gut microbiota (160). Enteric micro-organisms are important in mucosal immune cell development, DCs are capable of differentiating between pathogenic and commensal bowel flora. The specific microflora encountered can induce maturation and lead to T-cell driven inflammatory response. DCs must determine when to mount a protective or tolerant immune response (160). As UC is driven by a disproportionate inflammatory response in the absence of an obvious trigger, the tolerogenic properties of DCs may provide a therapeutic option. In our study, TCM (whether cultured in control or drug) reduced DC maturation markers when compared with media only. With the exception of two markers of maturation, there were no significant differences between DMSO, P3 and Q8. It is unclear why there was a lack of DC maturation in both the treatment and control groups. Both were cultured in 10% fetal calf serum (FCS), Penicillin, Gentamicin and Fungizone, perhaps this exerted an effect on DC maturation.

5.5.2 P3 treatment results in increased CD54 and PD-L1 compared with DMSO

Our aim in this chapter was to evaluate the interaction of our small molecule agents with DCs compared with DMSO. We sought to clarify whether these anti-inflammatory, anti-angiogenic agents exert significant effects on DC function and thereby influence immune response. We found that P3 treated DCs resulted in significantly increased expression of maturity markers CD54 and PD-L1. CD54, also known as intercellular adhesion molecule (ICAM)-1, has an important role in UC. CD54 controls the migration of neutrophils into the colonic mucosa. Genetic analysis has shown polymorphisms in the gene encoding CD54, this suggests that CD54 is significant in pathogenesis and perpetuation of the chronic inflammatory changes in UC (174). This hypothesis is supported by relative success in targeting CD54 blockade as a therapeutic option. Antisense oligonucleotide inhibitor of ICAM, Alicaforsen, has been shown to be effective in the treatment of distal UC (175). The increased presence of CD54 in the TCM treated with P3 is of uncertain significance.

The role of PD-L1 in UC is less clear. In our study we have shown P3 is associated with increased PD-L1 expression when compared with DMSO. PD-L1 and its ligands have a role in dysregulation of T helper response. In one study a significant increase in PD-L1 was found in active UC colonic explants. However increased PD-L1 secretion was found to suppress Th1 cell activity which led to reduction in inflammation (176). The same study showed that loss of PD-L1 expression in CD was associated with persistent

dysregulated Th1 induced inflammation. In our study P3 was shown to be associated with increased PD-L1 expression which may exert a protective effect in UC.

In this study we have shown that P3 treated DCs are associated with increased maturation markers, CD54 and PD-L1 when compared with DMSO. Q8 did not differ significantly from DMSO. The significance of these findings is uncertain given the evolving understanding of the role of DCs in UC. There were no other significant differences between the control and treatment groups, similar levels of maturation marker expression were noted and IL-10/IL-12p70 release did not vary significantly.

Chapter 6: Discussion

6.1 Aims of the thesis

1. Evaluate the colonic microenvironment in patients with UC, assess the expression of inflammatory and angiogenic analytes. Look for correlations between clinical parameters and analyte expression. Evaluate for crosstalk between inflammatory and angiogenic molecules.

2. Explore the therapeutic potential of two small molecule anti-inflammatory and anti-angiogenic agents in UC: Generate tissue cultured media and treat with control, P3 and Q8. Evaluate the ability of these drugs to reduce the over expression of inflammatory and angiogenic analytes with particular focus on key analytes relevant to UC pathogenesis.

3. Study the effects of P3 and Q8 on DC maturation and function. Compare maturation markers and IL-10/IL-12p70 release in DCs treated with P3 and Q8 to DMSO. Carry out further analysis on the effects of TCM on DC maturation and function, by comparing with media only.

6.2 Study application

At its core this thesis sets out to evaluate whether P3 and Q8 have potential as therapies for management of UC. The findings in this thesis support this hypothesis and both agents warrant further investigation.

6.3 Study limitations

There are limitations to this thesis;

1. The number of patients recruited is relatively small. Although numbers were sufficient to generate significant findings, analysis of the different patient subgroups (e.g. biologic naive/exposed) may have been affected.

2. In recent years, there has been a shift from treating to a specifically defined medical target, towards aiming to alleviate patient's symptoms. Increasingly there is more focus on patient reported outcomes (PRO) as measures in clinical trials. In this study a reduction in inflammatory and angiogenic analyte expression was taken as a positive outcome. Indeed key analytes targeted in the management of UC were significantly reduced. However, it is unknown whether reducing these analytes will produce an improvement in patient's symptoms.

3. Despite best efforts to maintain consistency in endoscopic reporting and histological assessment, some degree of inter-observer variability is inevitable. This has been minimised by incorporating a validated scoring mechanism (endoscopic mayo score) when reporting was carried out. Despite these limitations the data gained from this thesis is robust and both drugs warrant further work-up.

6.4 Areas for further research

1. These exciting anti-inflammatory and anti-angiogenic agents have significant potential for use in the management of UC. To date, colleagues in our unit have explored other possible roles for these agents and evaluated mechanisms of delivery. Both agents have been studied in CRC and oesophageal adenocarcinoma models.

2. The therapeutic target for P3 has not been fully elucidated. Quininib has been shown to inhibit cysteinyl leukotriene receptor 2. P3 appears to exert its effect independent of cysteinyl leukotriene receptor 2. Protein binding studies found <30% affinity, indicating P3 does not inhibit this receptor. Mass spectrometry testing is being carried out to identify molecular targets.

3. Further pharmacokinetic evaluation is required for both P3 and Q8, improved understanding of drug bioavailability is essential going forward. Data on gut wall metabolism, hepatic and renal clearance will allow improved understanding of these agents. Oral dosing is often the aim with newly developed medications as it is more convenient for the patient. These small molecule agents may have sufficient bioavailability to achieve plasma concentrations required, however further research is needed to accurately determine the pharmacokinetic profile.

4. The use of nanoparticles as a novel mechanism of drug delivery and enhanced therapeutic effect is a popular and growing field in research. Coupling P3 or Q8 gold nanoparticles may provide a synergistic effect. The use of P3 coupled with gold nanoparticles is currently being evaluated within our department.

5. In this study the effect of P3 and Q8 treated TCM on dendritic cells was evaluated. The secretion of IL-10 and IL-12p70 was measured via ELISA. Markers of maturation were assessed by flow cytometry, these provide a surrogate for DC function. It would be important to assess the effects of both drugs directly on DC function and T cells.

6.5 Summary of thesis findings

UC is a chronic inflammatory condition with a significant impact on patient's quality of life and a large economic burden. Targeting angiogenesis may provide a novel therapeutic pathway in the management of UC. Two novel small molecule agents used in this thesis were developed from parent drug quininib. These agents display potent anti-angiogenic and anti-inflammatory properties.

In this thesis the role of angiogenesis in the pathogenesis of UC was reviewed. In chapter one, pathological angiogenesis and its role in perpetuating the chronic inflammatory changes seen in UC were discussed. Angiogenesis is a process that in normal circumstances is tightly regulated, dysfunction in this regulation can cause angiogenesis to be continuously switched on. Chapter one highlighted that angiogenesis and chronic inflammation are inter-related. It has been hypothesised

that the vasculature plays a role in UC for over 50 years (177). The colonic distribution and often clear demarcation between normal and diseased colon support this hypothesis. Studies have shown that angiogenic molecules are found at increased levels in UC, VEGF was found to be significantly elevated in active UC (178). Despite robust evidence of a significant relationship between pathological angiogenesis and active UC there have been very few studies that have attempted to therapeutically target this pathway. This may be due to the side effect profile of many of the licensed anti-angiogenic agents. Targeting VEGF is associated with increased risk of gastrointestinal perforation and fistulae. These agents are used in the treatment of CRC where complications of a GI nature are more common, however evidence does suggest that anti-VEGF therapy increases this risk above what would be expected in a CRC population. No therapies for UC directly target angiogenesis, but infliximab has been shown to reduce VEGF-A by inhibition of leucocyte trafficking (74).This likely adds to its clinical efficacy. Other commonly used drugs like mesalamine and corticosteroids dampen angiogenic molecule release (179, 180). Angiogenesis plays a significant role in UC and may yield a novel therapeutic approach.

The two novel small molecule drugs used in this study were derived from parent drug quininib. Quininib was screened from a library of over 50,000 compounds and found to display significant anti-angiogenic activity in zebrafish. Two Quininib analogues were used in this thesis; P3 and Q8. Both these agents display potent anti-angiogenic and anti-inflammatory characteristics and as a result were labelled lead compounds and earmarked for evaluation in UC.

In chapter 3, clinical characteristics of a UC patient cohort were evaluated. The colonic microenvironment was investigated for angiogenic and inflammatory analyte expression. CRP is an acute phase inflammatory marker, in this chapter we showed that it correlated with disease severity. Interestingly, we also demonstrated that an elevated serum CRP correlated strongly with increased TNF-α in the colonic microenvironment. This is the first time this has been demonstrated and further strengthens the role for CRP to assess disease severity and therapy response in UC. Significant interrelationships between angiogenic and inflammatory analytes were noted. TNF-α correlated significantly with angiogenic analytes FLT-1 and bFGF. VEGF A was associated with increased inflammatory markers IL-2, IL-6, IL-10 and IL-13. This suggests that significant crosstalk exists between angiogenic and inflammatory analytes and supports the hypothesis that both are inextricably interlinked. There were also significant correlations between the patient's symptom profile and analyte expression. The partial Mayo score correlated with increased IL-6 and FLT-1 expression. This study demonstrated that increased disease duration was associated with increased angiogenic molecule expression (TIE-2 and VEGF C).

In chapter 4 both drugs were evaluated for their ability to suppress the release of key inflammatory and angiogenic analytes. Both P3 and Q8 led to a significant reduction in VEGF-A. VEGF-A is the most potent driver of angiogenesis and has been shown to be associated with increased disease activity in IBD (181). P3 also inhibited VEGF-C and PlGF when compared with control. Both P3 and Q8 had a significant anti-inflammatory

effect. Both agents suppressed the secretion of TNF-α, IL-2, IL-8, IL-12p70 and IL-13. TNF-α is an important therapeutic target in UC.

P3 displayed a significant anti-inflammatory effect in the biologic exposed patient group. These patients tend to have more severe disease and are more difficult to treat. P3 suppressed the release of IFN-γ, IL-4, IL-12p70, IL-13 and TNF-α when compared with control. This suggests that P3 may have a role in this difficult to treat subgroup of patients with UC who are biologic exposed.

P3 displayed an anti-angiogenic effect in the subgroup of patients with longstanding UC. These patients are at increased risk of CRC development by virtue of longstanding colonic inflammation. P3 caused inhibition of TIE-2, VEGF-C and PIGF. Anti-angiogenic treatments are employed in management of CRC to good effect. Targeting angiogenesis may provide protection against the development and spread of CRC. When both P3 and Q8 were compared in a head to head analysis they displayed similar efficacy. Neither agent significantly outperformed the other, although P3 did exert a reduction in PIGF when compared with Q8.

This study provides evidence that P3 and Q8 may have a role in the management of UC. Both molecules display strong anti-inflammatory and anti-angiogenic effects in the UC colonic microenvironment. These findings are consistent with previous data generated in zebrafish, CRC and oesophageal cancer explants.

Dendritic cells (DCs) are key cells in adaptive immunity. In chapter 5 the effect of TCM on DC maturation was evaluated and the impact of P3 and Q8 studied. Compared with media only, TCM appeared to inhibit DC maturation, all maturation markers with the exception of CD54 were significantly lower in the TCM groups. When evaluating the effect of each drug on DC maturation P3 was associated with increased expression of maturation markers CD54 and PD-L1, when compared with DMSO. Other markers of maturation were similar in each group, suggesting that neither drug significantly impacts on DC function.

6.6 Conclusion

Findings from this thesis have furthered the knowledge of angiogenesis in UC. The UC colonic microenvironment was characterised and significant correlations with clinical parameters identified. The role of CRP as a marker of disease severity was supported, and a previously unknown correlation between serum CRP and colonic TNF-α was discovered. This thesis identified crosstalk between inflammatory and angiogenic analytes, further supporting the hypothesis that pathological angiogenesis and chronic inflammation are inextricably linked. The role of two novel drugs in UC explants has been evaluated. Both agents have potent anti-inflammatory and anti-angiogenic properties. In this thesis the impact of P3 and Q8 on DCs and adaptive immunity was studied. P3 treatment was associated with an increase in markers of DC maturation (CD54 and PD-L1), no other significant differences were noted in DC maturation when compared with control. Both agents warrant further investigation and may become a therapeutic options for patients with UC.

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