Investigation of Novel Therapies to Modulate Inflammatory and Metabolism Profiles in the Ulcerative Colitis and Irritable Bowel Syndrome

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Cathy McShane

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Thesis Abstract

Inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) are chronic gastrointestinal disorders with overlapping pathophysiologies. Both conditions may be driven by a complex interplay of dysregulated immune responses, dysbiosis, environmental factors and impaired intestinal barrier function in genetically predisposed individuals.

This research aims to: (i) define the need for novel therapies in IBD; (ii) understand the pathogenesis of ulcerative colitis (UC) and IBS; (iii) investigate the mechanisms of action of infliximab in UC; and (iv) evaluate the therapeutic potential of novel natural plant extracts in UC and IBS. We hypothesise that metabolism significantly contributes to mucosal inflammation in these conditions and that natural plant extracts will exhibit anti-inflammatory effects via alteration of metabolic pathways in human colonic explants.

Despite advancements in IBD therapy with biologic and small-molecule agents, there appears to be a therapeutic ceiling of efficacy with single-agent therapy. A combination of two biologic therapies, or a biologic therapy and small molecule agent, with differing mechanisms of action, has the potential to improve IBD therapy outcomes. Information on the effectiveness and safety of this treatment strategy in IBD remains limited.[1-3]

Following the introductory Chapter 1, Chapter 2 reports a retrospective multicentre study of 109 IBD patients treated with combination biologic or small-molecule

therapies. Corticosteroid-free clinical response rates at 12 and 52 weeks were 39% and 38% respectively. Adverse events occurred in 26% of therapeutic trials and were predominantly disease-related. Combination therapy was moderately effective with an acceptable safety profile, highlighting the need for more effective strategies.

Chapter 3 reports assessments of inflammatory and metabolism profiles of healthy controls (HC), UC patients and IBS patients. Twenty-six patients who were undergoing colonoscopy were prospectively recruited in three cohorts (6 HC, 6 IBS, and 14 UC). Recto-sigmoid biopsies were collected, and ex vivo colonic tissue explants were generated and cultured for 24 hours. Baseline real-time energy metabolism profiles and secretion of ten inflammatory mediators were assessed.

We observed that UC and IBS patients exhibited distinct inflammatory and metabolic profiles compared to HC. Active UC was associated with elevated pro-inflammatory cytokines (IL-2, IL-6, IFN-γ, and IL-1β) and dysregulated energy metabolism, with shifts in energy production linked to disease duration. These findings identify novel therapeutic targets for UC.

Chapter 4 investigates whether these profiles were modifiable by an existing therapy (infliximab) and three natural plant extracts with therapeutic potential. Informed by traditional medicinal knowledge and in vitro screening for anti-inflammatory potential, these three plant extracts (*Cardamine pratensis* (cuckoo flower), *Potentilla erecta* (tormentil), and *Erica tetralix* (cross-leaved heath)) were selected for further exploration within the "Unlocking Nature's Pharmacy from Bogland Species" project, overseen by the NatPro Centre, Trinity College Dublin. Colonic explants

from each patient recruited in Chapter 3's study were co-cultured with and without treatments (infliximab, cuckoo flower, tormentil and cross-leaved heath).

Our study demonstrated that infliximab reduced TNF-α, IL-12p70, and IL-4 secretion in UC explants. Reduction in IL-4 following infliximab treatment was a novel observation and likely reflects the broader cytokine network modulation that occurs during treatment. The plant extracts exhibited pleiotropic effects, with cross-leaved heath reducing IL-4 and IL-6 in IBS explants, warranting further investigation for its potential application in gastrointestinal and allergy-related disorders.

In conclusion, this thesis underscores the need for innovative therapies in IBD and IBS, expands understanding of metabolism-inflammation interactions in UC, and identifies novel drug targets. The therapeutic potential of cross-leaved heath highlights the promise of natural extracts in addressing unmet clinical needs.

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Thesis Outputs

Publications

"Effectiveness, safety, and cost of combination advanced therapies in inflammatory bowel disease"

McShane C, Varley R, Fennessy A, Byron C, Campion JR, Hazel K, Costigan C, Ring E, Marrinan A, Judge C, Sugrue K, Cullen G, Dunne C, Hartery K, Iacucci M, Kelly O, Leyden J, McKiernan S, O'Toole A, Sheridan J, Slattery E, Boland K, McNamara D, Egan L, Ghosh S, Doherty G, McCarthy, J, Kevans D. Digestive and Liver Disease, Volume 57, Issue 1, 2025, Pages 274-281, ISSN 1590-8658. https://doi.org/10.1016/j.dld.2024.08.055.

Oral Presentations

"Natural Compounds in Ulcerative Colitis and Irritable Bowel Syndrome: Insights from Immune-metabolic Profiles" **McShane C,** O'Connell F, Piggott M, Obaidi I, Kevans D, Sheridan H, O'Sullivan J.

Moderated poster, UEG Week 2024.

Winner of National Scholar Award.

"Natural Compounds in Ulcerative Colitis and Irritable Bowel Syndrome: Insights from Immune-metabolic Profiles" **McShane C,** O'Connell F, Piggott M, Obaidi I, Kevans D, Sheridan H, O'Sullivan J.

The Irish Society of Gastroenterology summer meeting, 2024.

Awarded 1st place for best scientific abstract, Best Clinical & Scientific Abstract Session.

"Effectiveness, Safety, and Cost of Combination Advanced Therapies in Inflammatory Bowel Disease" **McShane C**, Varley R, Fennessy A, Campion J, Hazel K, Ring E, Marrinan A, Palmer C, Keogh A, Byron C, O'Sullivan A, Boland K, Buckley M, Dunne C, Farrell R, Hall B, Hartery K, Kelly O, Leyden J, McKiernan S, Moran C, Mulcahy H, O'Toole A, Patchett S, Sheehan D, Sheridan J, Slattery E, Smyth C, Ghosh S, McNamara D, Egan L, Doherty G, McCarthy J, Kevans D.

The Irish Society of Gastroenterology winter meeting, 2023

Selected for presentation in Top Abstracts - Presidents Choice oral presentation section.

Poster Presentations

"Combination biologic or small molecule therapy in Inflammatory Bowel Disease" **McShane C**, Varley R, Fennessy A, Campion J, Hazel K, Ring E, Marrinan A, Palmer C, Keogh A, Byron C, O'Sullivan A, Boland K, Buckley M, Dunne C, Farrell R, Hall B, Hartery K, Kelly O, Leyden J, McKiernan S, Moran C, Mulcahy H, O'Toole A, Patchett S, Sheehan D, Sheridan J, Slattery E, Smyth C, Ghosh S, McNamara D, Egan L, Doherty G, McCarthy J, Kevans D.

Poster presentation Disease Digestive Week, 2023. Gastroenterology, 2023-05-01, Volume 164, Issue 6, Pages S-1128-S-1129, 10.1016/S0016-5085(23)03605-3

"Effectiveness and Safety of Combination Biologic or Small Molecule Therapy in Inflammatory Bowel Disease" **C McShane**, R Varley, A Fennessy, J Campion, E Ring, O Kelly, E Slattery, G Doherty, J McCarthy, D Kevans.

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Abbreviations

Abbreviation	
ADL	Activities of daily living
ATP	Adenosine 5′-triphosphate
BCA	Bicinchoninic acid assay
CD	Crohn's disease
CGRP	Calcitonin gene-related peptide
CI	95% confidence interval
CM	Cultured media
CO2	Carbon dioxide
CRP	C-reactive protein
CSF	Corticosteroid free
DMF	Dimethyl fumarate
DMSO	Dimethyl sulfoxide
ECAR	Extracellular acidification rate
EDTA	Ethylenediaminetetraacetic acid
EIM	Extraintestinal manifestation
ELISA	Enzyme-linked immunosorbent assay

FODMAP	Fermentable oligosaccharides, disaccharides, monosaccharides and polyols
GDNF	Glial cell-derived neurotrophic factor
GI	Gastrointestinal
HC	Healthy controls
IBD	Inflammatory bowel disease
IBD-U	IBD-unclassified
IBS	Irritable Bowel Syndrome
ICU	Intensive care unit
IEC	Intraepithelial lymphocyte
IFN- γ	Interferon-γ
IFX	Infliximab
IL	Interleukin
ILCS	Innate lymphoid cells
IMID	Immune mediated inflammatory disorder
INITIATIVE	Investigator Network for Inflammatory Bowel Disease Therapy in Ireland
IQR	Interquartile range
JAK	Janus kinase
MAdCAM-1	Mucosal addressin-cell adhesion molecule 1

MCJ	Methylation-controlled J protein
MNPs	Mononuclear phagocytes
mtDNA	Mitochondrial DNA
mRNA	Messenger ribonucleic acid
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRX1	NOD [nucleotide oligomerization domain] like receptor X1
NMSC	Non-melanomatous skin cancer
OCR	Oxygen consumption rate
OCR:ECAR	OCR to ECAR ratio
OR	Odds ratio
O2	Oxygen
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
Pen-Strep	Penicillin-streptomycin
PMNs	Polymorphonuclear leukocytes
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
SCFA	Short-chain fatty acids
SGLT2	Sodium-glucose co-transporter 2
S1PR	Sphingosine-1-phosphate receptor

Spp	Species
Tcra -/-	T cell receptor alpha chain-deficient
T Helper	T helper
TGF-β	Transforming growth factor-beta
TNF- a	Tumour necrosis factor - α
UC	Ulcerative colitis
USA	United States of America
UST	Ustekinumab
VAT	Value-added tax
VIF	Variance inflation factor
5-ASA	5-aminosalicylic acid
5-HT	5-hydroxytryptamine
6EA	Cuckoo flower extract
26A	Cross-leaved heath extract
27B	Tormentil extract

Tables and Figures Legend

Tables:

Figures:

Chapter 1: Introduction

1.1. Inflammatory bowel disease

Inflammatory bowel diseases (IBD) are chronic, inflammatory conditions of the gastrointestinal (GI) system, classically comprised of Crohn's disease (CD) and ulcerative colitis (UC). They are typically characterised by chronic inflammation of the gut, and may be associated with extra-intestinal manifestations. The two conditions are thought to be distinct, if not discreet entities. Our understanding of the underlying pathophysiology of IBD is still lacking and treatment outcomes remain suboptimal.[4,5]

1.1.1. Crohn's Disease

CD affects the whole GI tract, from the mouth to the anus, in a discontinuous manner. It is characterised by transmural inflammation which may lead to complications, including fibrotic strictures, fistulas and abscesses. It is classified by the Montreal Classification system which helps categorises patients based on age of onset, and location and behaviour of disease.[4-6] (**Table 1**)

Montreal Classification of Crohn's Disease	
Age at diagnosis	A1: Less than 16 years
	A2: 17 – 40 years
	A3: Over 40 years
Location	L1: Ileal
	L2: Colonic
	L3: Ileocolonic
	L4: Isolated upper GI disease
Behaviour	B1: Non-stricturing, non-penetrating
	B2: Stricturing
	B3: Penetrating
	P: Perianal modifier

Table 1: Montreal Classification of Crohn's Disease

1.1.2. Ulcerative colitis

UC affects the colon, extending proximally in a continuous fashion. It is characterised by superficial mucosal inflammation. It causes ulcerations, bleeding and may give rise to fulminant colitis and toxic megacolon. The disease is classified by the Montreal Classification system which defines the disease by extent and severity.[6] (**Table 2**) The extent of UC colonic involvement influences the likelihood of requiring a colectomy. A systematic review reported a 19% 10-year rate of colectomy in those with extensive disease versus a 5% 10-year rate in those with proctitis. The extent can change over time in the absence or failure of treatment. 50% of those with rectosigmoid disease progress to extensive colitis over time.[5]

UC was selected as the primary disease model for IBD investigation for this thesis because it offers a more uniform platform for mechanistic and translational work compared to CD. Given that UC is confined to the colonic mucosa and extends continuously from the rectum, this reduces anatomical and histologic heterogeneity and facilitates consistent sampling of inflamed and non-inflamed tissue during colonoscopy which in turn increases the internal validity and statistical efficiency for detecting changes in the inflammatory and metabolic profiles.

Montreal Classification of Ulcerative Colitis	
Extent	E1: Ulcerative proctitis E2: Left-sided UC E3: Extensive UC (pancolitis)
Severity	S1: Mild UC *Four or fewer bloody stools daily, lack of fever, pulse of less than 90 beats/min, haemoglobin of 105 g/L or greater and ESR of less than 30 mm/h* S2: Moderate UC *Four stools daily but with minimal signs of systemic toxicity* S3: Severe UC *Passage of at least six bloody stools daily, pulse of at least 90 beats/min, temperature of at least 37.5°C, haemoglobin of less than 105 g/L and ESR of at least 30 mm/h*

Table 2: Montreal Classification of Ulcerative Colitis. ESR, erythrocyte sedimentation rate; UC, ulcerative colitis.

1.1.3. The rising burden of IBD

There is a growing incidence and prevalence of IBD worldwide. A 2022 population-based cohort study from Denmark showed that between 1995 and 2016 there has been a rising incidence of both CD and UC. The incidence rate (95% confidence interval) per 100,000 person-years rose from 9.1 (8.3–10.0) to 17.8 (16.8–19.0) for CD, and from 21.0 (19.8–22.3) to 28.4 (27.0–29.8) for UC in this time period. Children and young adults had the highest rates of increase in incidence. During this same period, the prevalence of IBD doubled. The highest increase in prevalence was seen in UC patients aged over 40 years where the prevalence increased by 2.5 fold.[7]

In Ireland, there is no database that collects accurate figures in relation to the incidence and prevalence rates of IBD in the population. However it is estimated that the disease affects at least 40,000 people nationally, and that 0.5% of the population have UC and 0.3% have CD. In 2011 there were 5.9 new cases of CD in Ireland per 100,000 population, and 14.9 new cases of UC per 100,000 population.[8]

Four epidemiological phases of IBD have been described. The first phase is termed 'emergence'. This phase reflects the period when sporadic cases of IBD began to appear in the western world following the industrial revolution. This is followed by the 'acceleration in incidence' phase: as industrialisation and urbanisation grows the incidence of IBD rises sharply, with prevalence remaining low. Newly industrialised countries in Asia and Latin America are currently in this phase. This is likely due to both improved detection rates and suspected environmental changes that occur with industrialisation. Most countries in the western world entered the third phase of

disease in the late 20th century: the 'compounding of prevalence' stage. This is characterised by a stabilised or declining incidence of IBD but rising prevalence due to the accumulation of cases over time and low mortality rates. The final, and as of yet theoretical, phase is that of 'prevalence equilibrium'. It is expected that prevalence will stabilise as incidence aligns with mortality, particularly in ageing populations.[9] These patterns of changes in disease incidence and prevalence globally reveal interesting clues to the aetiology of the disease. (**Figure 1**)

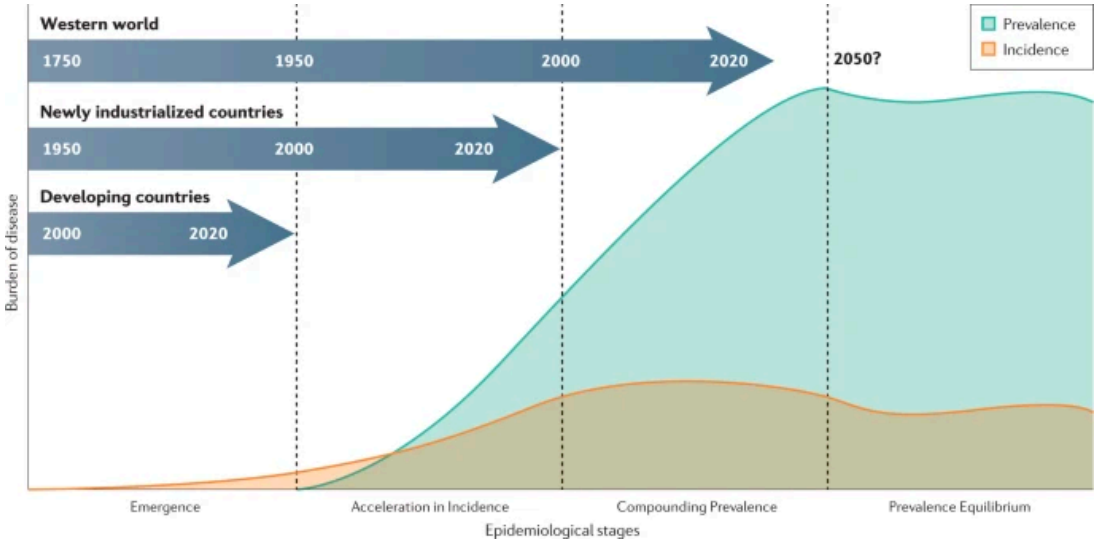

Figure 1. The four epidemiological stages of IBD evolution. IBD is hypothesised to evolve within four epidemiological phases. The orange line represents incidence, and the blue line represents prevalence. In developing countries, sporadic cases with an associated increase in incidence of IBD is noted (emergence phase). Recently industrialised countries then enter the acceleration of incidence phase, where incidence rises but prevalence remain low. Following this, there is a compounding of prevalence phase, where incidence is stable but prevalence increases. Western countries are currently within this phase. The final phase is termed prevalence equilibrium. This is a hypothetical phase that western countries are expected to enter within this century. This will be seen when incidence matches mortality rates, and prevalence will therefore remain static. Figure attributed to Kaplan & Windsor, Nature Reviews Gastroenterology & Hepatology.[9]

1.1.4. The aetiology of IBD

The exact aetiology of IBD remains unknown, however it is likely multifactorial involving a complex interaction between environmental, microbial and immunological factors in a genetically predisposed individual.

There is a higher incidence of IBD in first degree relatives of those with IBD, suggesting some genetic basis to risk of disease. There is a greater heritable risk associated with CD than UC. Genome-wide association studies have identified over 240 IBD risk variants that are associated with a range of processes including intestinal barrier function, regulation of innate and adaptive immunity, microbial sensing, and the autophagy pathway. However, despite this only 8-13% of CD and 4-7% of UC can be explained by known IBD risk loci, with higher rates seen in paediatric onset IBD.[4,10]

As suggested by the rise in incidence of IBD associated with industrialisation, environmental triggers have been associated with pathogenesis of the disease. Recent work from the Crohn's and Colitis Canada-Genetic, Environmental, Microbial project, a prospective cohort study of first-degree relatives of patients with CD, has revealed multiple environmental risk factors. Early life exposure to a parent with CD increases the risk of IBD development in the offspring. Protective factors include living with a dog between the ages of 5 to 15 years, and living with a large family size in the first year of life.[11,12] Additional early life exposures found to increase risk by metanalysis include prenatal exposure to antibiotics, exposure to tobacco smoke and development of otitis media infection, whereas breast feeding

had a negative correlation with the risk of IBD.[13] Antibiotic exposure later in life also increases risk of developing IBD, especially exposure in those older than 40 years.[14]

These risk factors suggest that dysbiosis may be associated with risk of developing IBD. The gut microbiome is established at birth, with key commensals acquired from the mother. It undergoes a major shift within the first two years of life, becoming more diverse and similar to that of an adult. It is hypothesised that disruption of microbiome development during a critical period within the first few years of life leaves a deficit in host-microbial cross-talk leading to an increased risk of IBD. However there is currently insufficient data to draw definitive conclusions in relation to this theory.[8,15-17]

There is a symbiotic relationship between intestinal microbes and the host. Microbes are involved in the metabolism and fermentation of dietary factors. In doing so they produce short-chain fatty acids (SCFA). These SCFA play a key role in maintaining intestinal barrier function, the first line of defence to potential pathogens. Disruption in barrier function allows invasion of these pathogens and subsequent inflammation. Microbes also play an immunomodulatory role. A multitude of studies have shown that both CD and UC are associated with dysbiosis. Changes in the abundance of multiple taxa have been associated with risk of developing CD including *Ruminococcus torques*, *Blautia*, *Colidextribacter*, and *Roseburia.* The exact role these changes play during the transition from health to disease has yet to be elucidated.[4,17,18]

Perturbations across the cross-kingdom microbiome (bacteria, fungi and viruses) are tightly linked to IBD pathogenesis. Beyond simple "dysbiosis," contemporary multi-omics shows a shift toward functional changes: loss of SCFA-producing taxa (for example *Faecalibacterium prausnitzii*, *Roseburia*) and gain of pathobionts (for example adherent-invasive *E. coli*), with fluctuations that track disease activity. Fungal and viral communities also contribute (for example strain-dependent *Candida albicans* toxins, and virome changes). Microbial metabolites, including SCFAs and secondary bile acids, shape epithelial barrier integrity and Treg/TH17 balance.[19]

Mucosal immunity within the GI tract strikes a balance between protection against pathogens and immune tolerance to commensal microbes and dietary antigens. Both innate and adaptive immune responses can become dysregulated in IBD causing excessive proinflammatory cytokine production with alterations in homeostatic pathways.[4]

1.1.5. Presentation and diagnosis of IBD

Both CD and UC can present in a similar fashion with symptoms that may include diarrhoea with or without blood, abdominal pain, bloating, fatigue and weight loss.[20] This generally occurs in a relapsing and remitting fashion. These symptoms prompt investigation including ileocolonoscopy and biopsy, and small bowel imaging studies if CD is suspected.

Distinguishing between the two diseases can pose a significant clinical challenge. However the histopathological hallmarks can differ between both diseases and can help in differentiating them. CD classically shows patchy inflammation, typically worse in the proximal colon if there is colonic involvement. There may be crypt distortion and granuloma formation. Granulomas only occur in 50% of CD patients. UC on the other hand is favoured when diffuse inflammation is seen. There are no pathognomonic hallmarks of UC histologically. Basal plasmacytosis, diffuse crypt atrophy and distortion, villous surface irregularity and, mucus depletion all point towards UC in the right clinical context. Partially treated UC can become patchy in distribution and backwash ileitis can occur. On occasion cryptolytic granulomas can occur in UC.

It is not possible to distinguish between UC and CD in 5-15% of patients. These individuals are ultimately diagnosed with IBD-unclassified (IBD-U). Research suggests that roughly 3% of those initially diagnosed with UC get reclassified as CD during their disease course, whereas less than 3% of CD get reclassified as UC.[5,21]

1.1.6. Current treatment strategies for IBD

Over the past two decades our understanding of the pathogenesis of IBD has increased greatly. In tandem with this there have been many new targeted therapies brought to market. The choice of therapy depends on disease severity, location, clinical factors and treatment history.

Mild to moderate UC is treated first line with 5-aminosalicylic acids (5-ASAs) for induction and maintenance of remission. The mechanism of action of 5-ASAs remains unknown, however their use in IBD is long established. Rates of clinical remission after 6-8 weeks of treatment range from 35-64%. Moderate to severe UC generally requires escalation of therapy. Remission historically has been induced with corticosteroids which act as a bridge to maintenance therapy. However there are some newer therapies that show good efficacy in induction of remission. Advanced therapies for maintaining remission include biologic monoclonal antibodies against tumour necrosis factor alpha (TNF) (i.e. infliximab (IFX), adalimumab), α4β7 integrins (i.e. vedolizumab), interleukin (IL) 12 and IL-23 (ustekinumab), and IL-23 p19 subunit (i.e. risankizumab, mirikizumab), and oral small molecules that inhibit Janus kinase (i.e. upadacitinib, filgotinib, tofacitinib) or modulate sphingosine-1-phosphate (i.e. ozanimod). Previously relied upon immunomodulators are now rarely used for UC monotherapy due to their safety profile. However they continue to play a role in combination therapy, whereby they reduce immunogenicity to anti-TNF therapies.[5,22]

Surgical colectomy is reserved for those refractory to medical therapy or due to the occurrence of a UC related complication (haemodynamic instability, toxic megacolon, perforation). Rates of response to advanced therapies are consistently 30 – 60% across clinical trials, with absolute rates of clinical remission no more than 25% greater than with placebo.[23] However despite novel targeted pathways and drugs developed, meta-analysis has shown that the 10 year colectomy rate remains at almost 10%.[22,24-26]

Treatment of CD follows a similar pattern as UC. Remission is generally induced by corticosteroids. Maintenance therapy is essential to prevent inflammation and resultant stricturing and penetrating complications. The armamentarium of advanced therapies broadly mirrors UC, as do efficacy outcomes. The PROFILE Trial from the United Kingdom recently confirmed that early, top-down therapy (with combination IFX plus immunomodulator) was superior to accelerated step-up of conventional therapies in newly diagnosed active Crohn's disease and associated with superior outcomes at one year.[27] However, despite the recent therapy advances, most CD patients will require surgical resection at some stage during their disease course.[28] This highlights the necessity for early access to advanced IBD therapies.

The STRIDE-II guidelines recently confirmed the long-term treatment targets for IBD. These include: clinical remission, endoscopic healing, restoration of quality of life and absence of disability. There remains uncertainty regarding the benefit of targeting histological and transmural healing in UC and CD respectively.[29] The VERDICT trial, which assesses the benefit of histologic remission compared to endoscopic remission alone in UC, is expected to clarify the treatment target endpoint upon its publication (NCT04259138).[8] Whereas, the VECTORS study aims to clarify if transmural healing, as assessed via intestinal ultrasound, is a superior treatment target to endoscopic remission in CD (NCT06257706).[8]

IFX, a monoclonal anti-TNF with established efficacy in UC, is included as a reference therapy to benchmark the natural-product experiments in UC and IBS.[30] While IFX's clinical and anti-inflammatory effects are well defined, its mucosal metabolic effects are less clearly characterised.[31] Using IFX ex vivo provides a

positive control for the explant platform and defines a pre-specified cytokine release profile. We use this signature to compare the effects of plant-derived extracts.

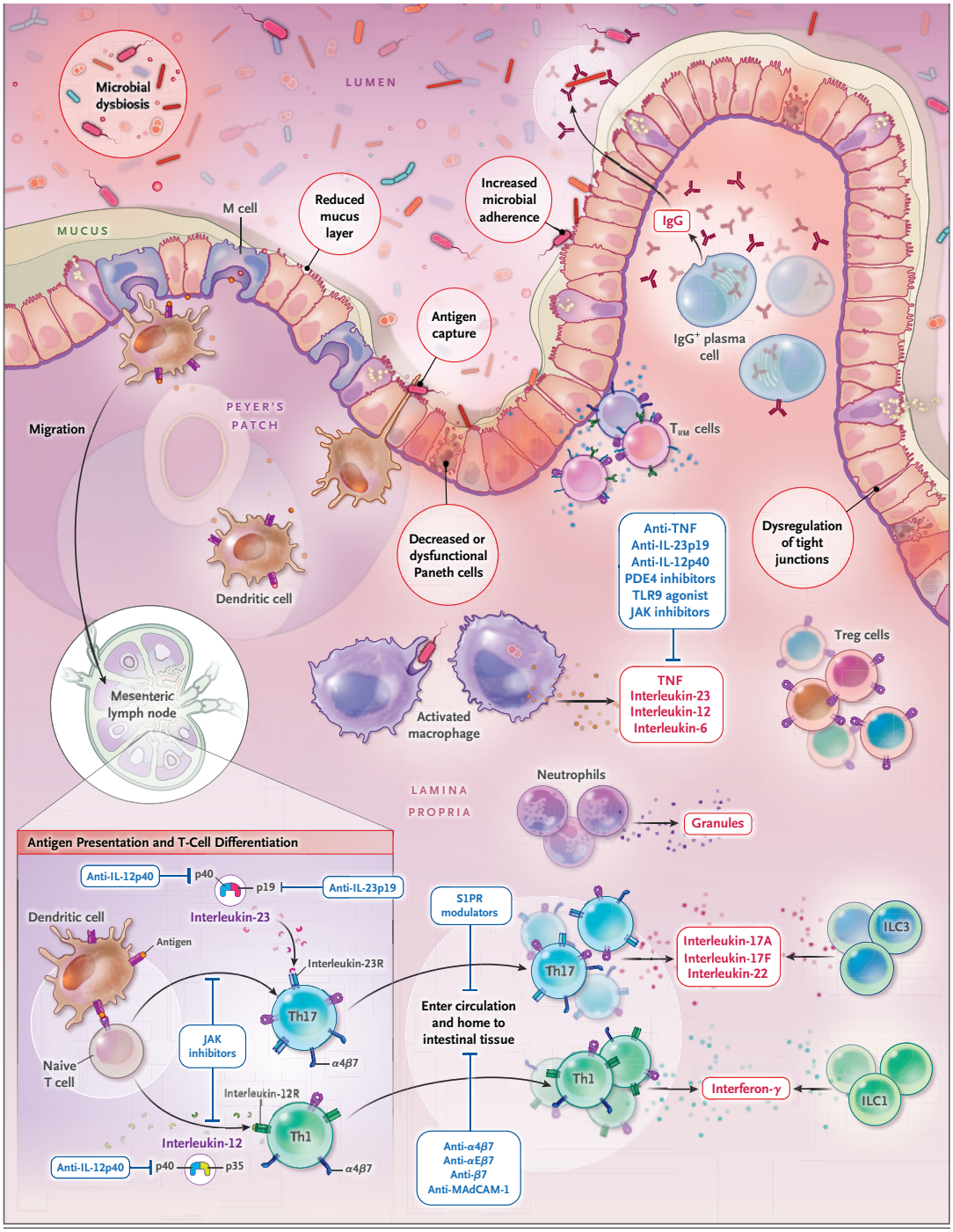

Figure 2. The dysregulated mucosal immune system in IBD and associated treatment targets. IBD occurs due to a complex interplay between dysbiosis, a disruption in the intestinal mucus layers and epithelial tight junctions, abnormal Paneth cells and increased intestinal permeability. This causes increased exposure of the intestinal immune cells to potential pathogens, causing an inflammatory response. Macrophages engulf the bacteria and release inflammatory cytokines such as TNF, IL-6, Il-23, and IL-12. Dendritic cells may stay in Peyer's patches or migrate to mesenteric lymph nodes where they present antigens to Naïve T cells which then differentiate into T helper 1 (Th1) or T helper 17 (Th17) cells. These may enter systemic circulation and home to intestinal tissue producing inflammatory

cytokines; INF-γ by Th1 cells and IL-17A, Il-17F, and IL-22 by Th17 cells. There is an increase in innate lymphoid cells groups 1 and 3 also, which produce INF-γ, and IL-17A respectively. Plasma B cells are also increased and produce IgG. Tissue-resident memory T cells in the epithelium and lamina propria also produce inflammatory cytokines, killing infected cells, alerting innate cells, and recruiting additional immune cells. The blue boxes represent licenced and experimental therapies for IBD. These include therapies that block the inflammatory cytokine cascade (anti-TNF, anti-IL-12 antibodies), inhibit signal transduction cascades downstream of inflammatory pathways (JAK inhibitors), block lymphocyte trafficking to the intestine (anti-α4β7, anti-β7, anti-αEβ7, and MAdCAM-1 antibodies) or inhibit lymph-node egress (S1PR modulators). IL, interleukin; INF, interferon; JAK, Janus kinase; MAdCAM-1 Mucosal addressin-cell adhesion molecule 1; S1PR, Sphingosine-1-phosphate receptor; Th, T helper; TNF, tumour necrosis factor. Figure attributed to JT Chang, New England Journal of Medicine.[4]

1.1.7. Biochemical biomarkers in IBD

C-reactive protein (CRP) is a plasma protein, predominantly synthesised by the liver, that is an acute phase reactant. Its production is stimulated largely by IL-6, and to a lesser degree by IL-1β and TNF-α. Following activation and binding of the ligand, it stimulates the complement cascade and phagocytosis. CRP rapidly increases with infection or inflammation and has a short half-life which makes it a useful biomarker to assess both infective and inflammatory diseases and monitor their response to therapy.

The use of CRP as a biomarker in IBD has long been established. However there is great difference in the CRP response to disease activity in CD compared to UC. CRP levels correlate well with disease activity in CD but only modestly at best with disease activity in UC. The reasons for this difference are not fully defined but likely include the transmural inflammatory distribution and higher systemic levels of IL-6 associated with CD.[32-34]

Faecal biomarkers of disease activity have higher specificity and sensitivity for intestinal inflammation in IBD than traditional serum biomarkers. Faecal calprotectin has emerged as the leading faecal biomarker in current clinical practice, correlating significantly with clinical and endoscopic disease activity in CD and UC. Calprotectin is cytosolic protein complex (comprising of S100A8 and S100A9) that is mainly expressed in neutrophils. It activates innate immune responses, promoting the secretion of pro-inflammatory mediators such as IL-1β, IL-6, and TNF-α and reactive oxygen species. Calprotectin performs well in distinguishing IBD from IBS,

with a negative predictive value of 99.8%, and a positive predictive value of 9%. Unlike CRP, it performs better in UC compared to CD in predicting endoscopic disease activity.[34-37] However, it is notable that other conditions can give rise to an elevated calprotectin, especially other inflammatory diseases such as infectious colitis and colorectal cancer and drugs such as nonsteroidal anti-inflammatories.[38]

1.1.8. Morbidity and mortality in IBD

IBD is associated with significant morbidity. As described above, patients may develop disease related complications, including toxic megacolon, perforation, and stricturing, and penetrating complications. These lead to hospitalisations, surgeries, and reduced quality of life.[22,39]

IBD-associated dysplasia remains a risk. IBD patients have a 1.4–1.7 higher risk of developing colorectal cancer compared to the general population. It is suspected that unbridled chronic inflammation causes this increased risk.[40]

There are conflicting reports regarding the impact of IBD on mortality. However multiple population based studies have suggested a small increase in mortality.[41-43] A recent global study, collecting data from 204 countries, between 1990 to 2021, reported that disease-adjusted life years and mortality have decreased.[44] The lasting impact of advanced therapies for IBD on morbidity and mortality as of yet remains undefined.

1.1.9. Precision medicine

Despite many new therapies for IBD coming to market over the past two decades, which incorporate novel mechanisms of targeting the disease, our ability to select a therapy in a biology-driven manner remains crude. The response rates to therapy remain suboptimal, as determined by the efficacy rates seen in clinical trials and real-

world outcome studies where up to 30% of patients are primary nonresponders to initial treatment and up to 50% of patients become secondary nonresponders. IBD patients form a heterogenous cohort, often with unpredictable responses to therapies. Precision medicine strives to overcome this therapeutic ceiling in treatment efficacy by tailoring an individual's care with a biomarker approach to better risk stratify and guide therapy depending on a person's unique characteristics.[45,46]

The discipline of precision therapy is in its infancy in IBD. However we can learn from the field of oncology where it is already being successfully applied in clinical practice. Although there are differences in the disease nature between the two, whereby cancer is typically clonal and driven by molecular aberrations, and IBD is due to multifactorial triggers with greater heterogeneity, oncology treatment outcomes have greatly benefited from detailed profiling of an individual's disease and the application of a similar approach might benefit IBD care. This includes next-generation sequencing, allowing clinicians to select a treatment on a biological basis. Benefits of precision medicine in oncology care have only become a possibility due to the greater choice of targeted drugs available to treat cancer. As such if we are to move away from the untargeted phenotypically driven approach currently employed in treating IBD towards molecular profiling of disease with targeted treatments, novels therapies will need to be developed.[45,47]

Preclinical models to profile disease are key to developing the field of precision medicine in IBD. One such approach which has been employed in drug development in oncology is the patient derived explant model. Patient derived explants involve

the ex-culture of fresh disease tissue samples, therefore maintaining the tissue architecture and proliferative capacity of the tissue. It allows detailed assessment of the disease microenvironment on an individual basis. It can be used for drug development, with good predictive value of in vivo response. The use of patient derived explants in IBD is growing where it has been used for both drug development and biomarker discovery.[48-50]

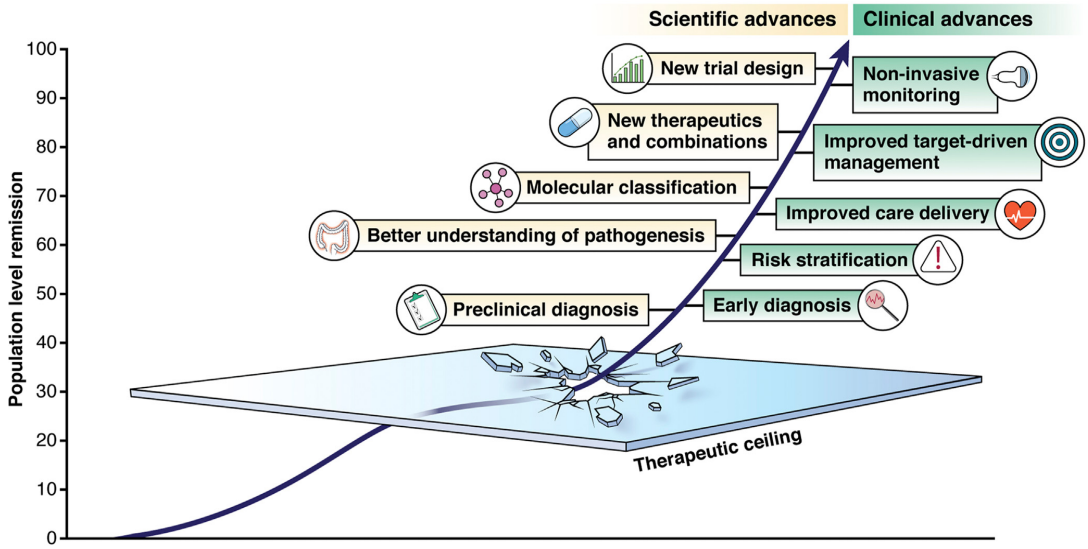

Figure 3. Breaking the therapeutic ceiling of drug efficacy in IBD. There appears to be a ceiling of treatment efficacy across all clinical trials for novel IBD therapies, with real-world remission rates being maximally 30-60% of the treated population. To overcome this a combination of scientific and clinical advances is required to allow discovery of new therapeutic treatment targets and increased accuracy of treatment selection for an individual. Figure attributed to Raine & Danese, Gastroenterology.[51]

1.2. Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) is one of the most common GI diseases encountered in both primary and secondary care. It is estimated to affect 1 in 10 people. It is a disorder of the gut-brain axis.

IBS is characterised by abdominal pain associated with a change in bowel habit. It is diagnosed using the Rome IV diagnostic criteria: "recurrent abdominal pain on average at least one day/week in the last three months, associated with two or more of the following features: related to defecation, associated with a change in frequency of stool, or associated with a change in form (appearance) of stool".

There are four subtypes of disease: IBS with predominant constipation, IBS with predominant diarrhoea, IBS with mixed bowel habit, and IBS unclassified. Criteria must be fulfilled for at least 3 months with a symptom onset of at least 6 months prior to diagnosis. Inflammatory intestinal diseases should be ruled out by faecal calprotectin, C-reactive protein (CRP) and erythrocyte sedimentation rate testing, and coeliac serology should be negative.[8,52]

The prevalence of IBS varies significantly depending on geography and the diagnostic criteria used. A Rome Foundation study using the Rome IV diagnostic criteria found global prevalence to be 4.1%, with higher rates seen in females.[52] Although rates vary globally the impact of IBS in terms of economics and quality life are thought to be comparable worldwide.[53]

1.2.1. Aetiology of IBS

The pathophysiology of IBS is poorly understood. It is thought to be due to a complex interplay between the gut-brain axis, genetics, microbiome, psychological factors, and in some cases infections. Symptoms are often triggered by stress or an infection in a genetically susceptible person. Post-infectious IBS occurs following infective gastroenteritis and may last many years. A broad range of pathogens have been implicated including common pathogens like *Campylobacter jejuni* and *Escherichia coli*.[52]

Similarly to IBD, increased intestinal permeability is thought to be an early event in the pathophysiology of IBS. This mechanism has been most described in post-infectious IBS or IBS diarrhoea predominant. Biopsies from patients with IBS diarrhoea predominant have been shown to have increases spaces between epithelial cells, with an associated increase in translocation of macromolecules through the intestinal mucosa. The exact cause of this "leaky gut" is unknown but there is evidence to support that genetic, epigenetic, microbial and food allergies all play a role. One study suggests over 50% of IBS patients have non-IgE mediated food allergies, causing damage to the intestinal barrier when exposed to the food antigen.[53-55]

There are dysregulated neuroimmune interactions associated with IBS. Animal models have demonstrated that mucosal mediators isolated from IBS patients show increased activation of visceral and somatic pain pathways when applied to rodent intestinal tissue. Mast cells and enteroendocrine cells are thought to be implicated in

this abnormal signalling. Higher numbers of degranulating mast cells are found in IBS tissue samples compared to healthy controls. There is also in increase in mast cell signalling mediators including histamine and proteases. These in turn have been shown to enhance sensory neuron excitability, contributing to visceral hypersensitivity.[55]

Genetic and epigenetic findings support current theories of IBS pathogenesis that implicate reduced intestinal barrier function, dysregulated immune response and abnormal neuronal signal transduction. Genetic abnormalities of the serotonin reuptake transporter have been widely studied. A meta-analysis including 27 studies found that IBS constipation predominant was associated with a serotonin reuptake transporter polymorphism. Other genetic variants implicated in IBS are CLDN1 (Claudin-1) and CDH1 (Cadherin-1) which regulate intestinal permeability and epithelial cell adhesion, respectively. As such, variants in these genes may contribute to the "leaky gut" phenomenon.[55]

The intestinal microbiome of IBS has been shown to be different than that of healthy controls, with reduced diversity and alterations in abundance of specific taxa. *Ruminococcus torques* has been shown to be increased whereas two groups of uncultured Clostridiales are reduced. Alterations in these taxa have been found to correlate with symptoms. When microbiota derived from IBS patients are used to colonise the gut of germ-free mice visceral hypersensitivity, impaired intestinal permeability and changes in gastrointestinal transit time are evoked. This underpins the potential aetiological role of the microbiome in IBS.[55]

Patients often report dietary triggers for their symptoms. A diet low in fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAP) is recommended as a therapeutic intervention. Foods high in FODMAPs are hypothesised to trigger symptoms through increased fermentation and gas production by gut bacteria. This further suggests the role dysbiosis may play in IBS.[8,55,56]

Psychological comorbidities, such as anxiety and depression, are prevalent in IBS and influence symptom perception and severity through the gut-brain axis. These interconnected factors highlight the complexity of IBS and the need for individualised management approaches.[56-59]

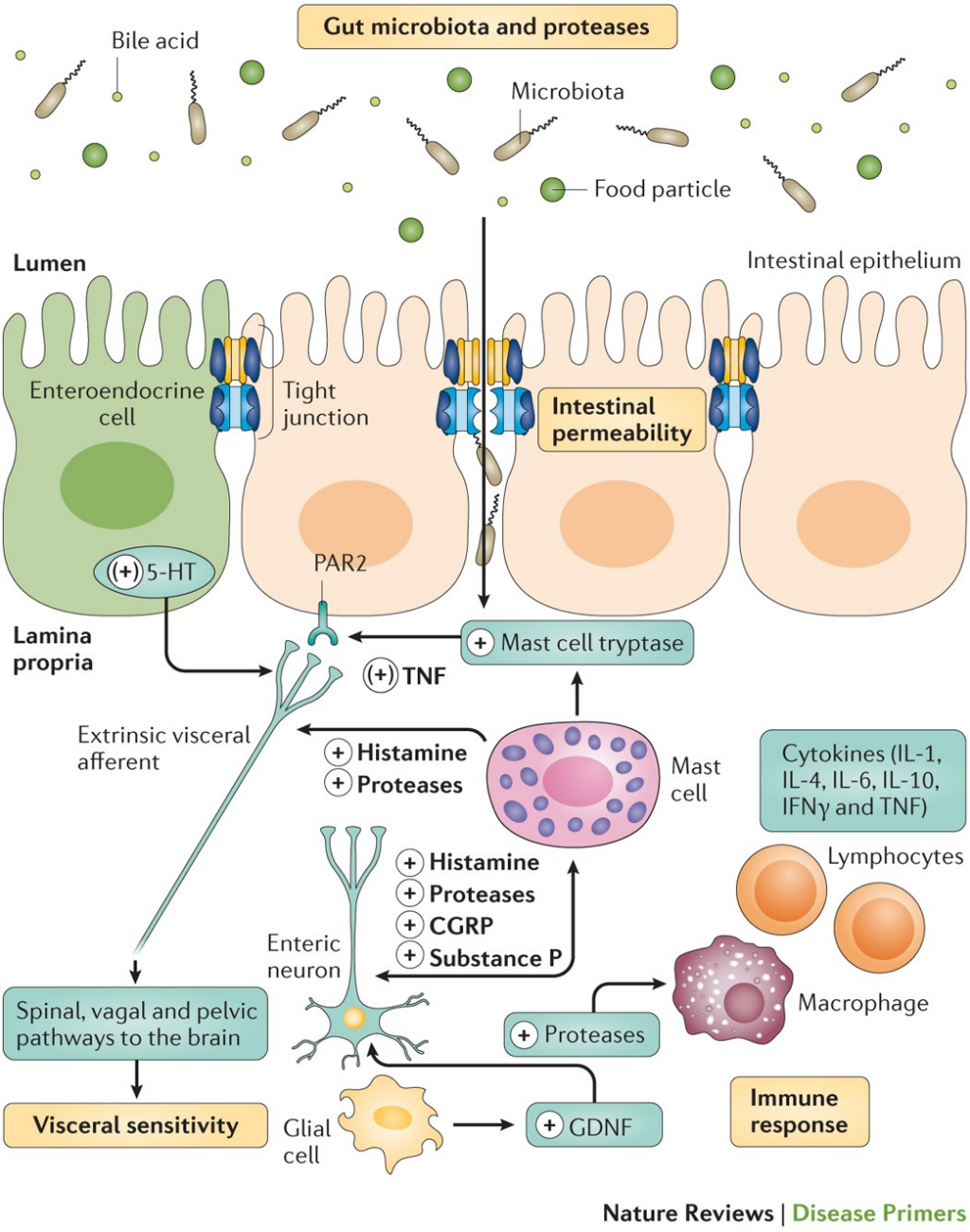

Figure 4. The pathophysiology of IBS. The cause of IBS is not fully understood. However dysbiosis, intestinal permeability, immune activation, and sensitivity of the enteric nervous system have all been implicated. This figure shows those mediators that are likely involved in IBS pathogenesis, with the plus symbol representing activation of the target cell. Factors without brackets have been demonstrated in human tissue and those with brackets in animal models. 5-HT, serotonin; CGRP, calcitonin gene-related peptide; GDNF, glial cell-derived neurotrophic factor; IL, interleukin; PAR2, proteinase-activated receptor 2; TNF, tumour necrosis factor. Figure attributed to Enck et al, Nature Review Disease Primers.[58]

1.2.2. Current treatments for IBS

The goal of IBS treatment is symptom control. Treatments are selected depending on the subtype of disease. First line management for all IBS patients includes basic dietary advice, regular exercise, and a probiotic. Further first line therapies are dependent on IBS subtype; loperamide for diarrhoea, laxatives for constipation, and antispasmodics for mixed or unclassified IBS.[52]

Second line dietary interventions include a diet low in fermentable oligosaccharides, disaccharides and monosaccharides and polyol. Second line pharmacological therapies for abdominal pain include gut-brain neuromodulators such as serotonin reuptake inhibitors or tricyclic antidepressants. Rifaximin, a non-absorbable antibiotic is an effective second line therapy for IBS diarrhoea predominant, as is eluxadoline, a mixed opioid receptor drug, and 5-Hydroxytryptamine 3 receptor antagonists. IBS constipation predominant second line pharmacological therapies include guanylate cyclase-C agonists, and chloride channel activators. A range of psychological therapies with low quality supporting evidence can be employed including IBS-specific cognitive behavioural therapy and gut-directed hypnotherapy.[52] Overall the response to treatments is variable, with symptoms persisting beyond a year in about 75% of cases.[53]

1.2.3. IBS related morbidity

Although IBS has not been associated with excess mortality, it causes significant individual and societal morbidity.[60] The economic cost of IBS is high, with estimates ranging from £45.6–200 million per annum in the UK. Patients often find it difficult to work: it is estimated that between 5-50% of people with IBS require time off work due to symptoms.[53] Results from the LOGIC study show IBS patients have a significant symptom burden, impaired health-related quality of life, reduced work productivity, and a low overall health status.[61]

1.3. IBS–IBD overlap

A 2012 meta-analysis which comprised 13 studies that examined IBS symptoms of UC and CD in remission found that 36% of UC patients and 46% CD patients reported IBS-like symptoms, with a female predominance. Higher levels of anxiety were reported in these patients. Endoscopically IBD and IBS look very different with IBD showing overt inflammation and IBS showing, normal healthy mucosa. Histological analysis of IBS mucosa can show an increase in inflammatory cells including lymphocytes and mast cells. This and other similar aetiological factors such as dysbiosis suggest that there may be some overlapping pathophysiology of disease processes.[59,62] (**Figure 5**)

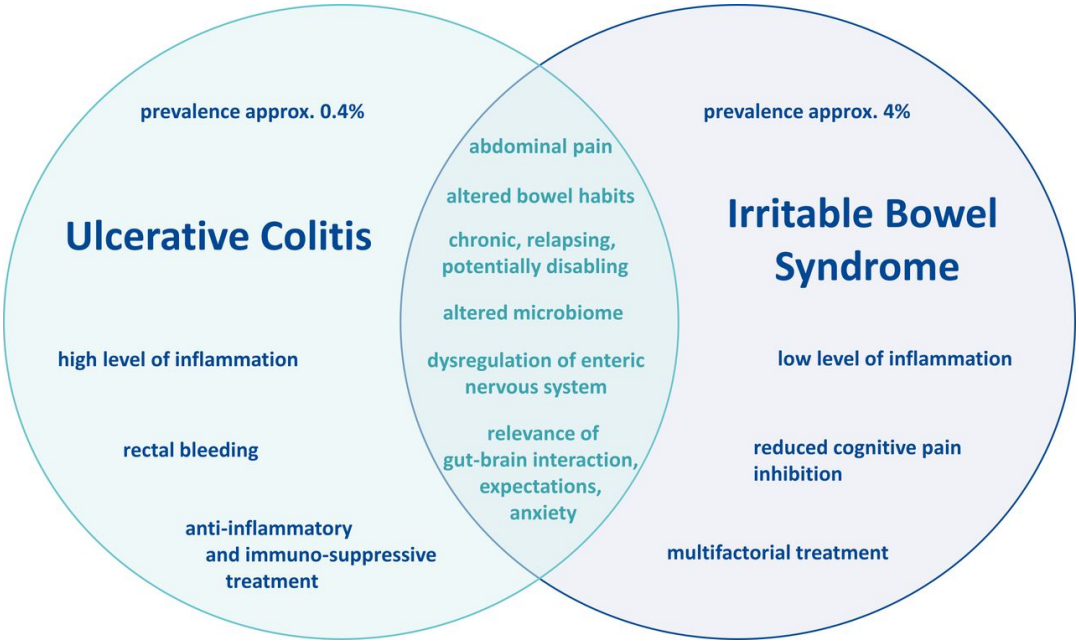

Figure 5. UC and IBS have many overlapping features but are different entities. UC has a prevalence of roughly 0.4% of the population compared to a prevalence of 4% in IBS. Both diseases can cause abdominal pain, altered bowel habits, and are chronic, relapsing and potentially disabling conditions. There are shared aetiological factors including dysbiosis, dysregulation of the enteric nervous system and abnormalities of the gut-brain axis. UC patients have a high inflammatory burden, rectal bleeding and are treated by anti-inflammatory and immunosuppressant medications. IBS patients have low inflammatory burden, reduced cognitive pain inhibition, and treatments are broad ranging and often multimodal. IBS, irritable bowel syndrome; UC, ulcerative colitis. Figure attributed to Löwe et al, BMJ Open.[55]

1.4. Immunometabolism

Immunometabolism refers to the interaction between the immune system and metabolic processes, highlighting the bidirectional regulation where metabolism influences immune cell function, and immune responses affect metabolic pathways. Cellular metabolism involves complex biochemical reactions that create energy and molecules that act on the microenvironment, regulating cellular activation and interaction. [8,63,64] Mitochondria, membrane-bound organelles, play an integral role in cellular metabolism through their role in providing energy to these cells via oxidative phosphorylation. They also produce reactive oxygen species, reactive nitrogen species, induce cell death and transduce stress and metabolic signals, thus playing a key role in homeostasis. Further to this, they play a key role in the metabolism (via β-oxidation) of short chain fatty acids (SCFA). These SCFA are mostly produced by fermentation of fibre by gut microbes. They play a significant role in maintaining barrier function, the principal line of host defence to potential pathogens in the luminal environment and serve as a key mediator between the gut microbiome and mitochondria.[65]

The Warburg effect, initially reported by Otto Warburg in the 1920s, describes the ability of cancer cells to metabolise glucose anaerobically via glycolysis even when oxygen is available. During inflammation, metabolic reprogramming occurs in a similar fashion, whereby cellular metabolism shifts to rely on glycolysis to provide energy to pro-inflammatory cells such as M1 macrophages and T helper 17 cells. Reprogramming immune cells away from inflammatory phenotypes via targeting these metabolic processes has become a treatment target in immune conditions. This approach is likely to avoid side effects associated with systemic immunosuppression as it specifically inhibits cells with an increased metabolic demand.[66-68]

1.4.1. Immunometabolism in IBD

Immune cells such as T cells, macrophages, dendritic cells, and natural killer cells have all been shown to have altered metabolic profiles in IBD. IBD patients have been found to have reduced ATP levels within the intestinal mucosa suggesting cellular stress and bioenergetic failure of mitochondria in IBD. Genome-wide association studies suggest that 5% of IBD susceptibility genes have key roles in regulating mitochondrial [69-71]

It is not known if mitochondrial dysfunction seen in IBD is primarily due to inflammation or if it plays a role in the pathogenesis of disease. However, UC colonic epithelial cells have been shown to have pathological alterations of mitochondrial ultrastructures even before the global changes and inflammation are seen, thus suggesting they are involved with the pathogenesis of disease.[72] When mitochondrial damage occurs an energy deficit state ensues, increasing cell death sensitivity, and impairing intestinal barrier function. IBD patients' immune and epithelial cells show downregulated mitochondrial metabolism genes compared to healthy controls. Supporting this finding, enterocytes extracted from IBD tissue show abnormally swollen mitochondrial with irregular cristae. With these changes comes a reduction in adenosine 5′-triphosphate (ATP) production and an increase in reactive oxygen species (ROS).[73]

Targeting dysregulated metabolic processes to repolarise inflammatory cells into an anti-inflammatory state has become a focus of IBD drug development. Phase 1b results have recently been published about the use of the small molecule NX-13, a NLRX1 (NOD [nucleotide oligomerization domain] like receptor X1) agonist, in

active UC. NLRX1 is a negative regulatory NOD-like receptor found on the mitochondrial membrane of most cells. Unlike most other NLRs which are pro-inflammatory, NLRX1 is immunoregulatory.[74] It inhibits nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and interferon-γ (IFNγ), key players in the pathogenesis of inflammation. Results from this study have shown an acceptable safety profile. [75,76]

Interestingly, two studies have linked colonic inflammation and mitochondrial dysfunction in the hippocampus, a region critical for mood regulation. Mouse models show that colitis induces mitochondrial dysfunction in the hippocampus, correlating with anxiety and depression-like behaviour. This dysfunction was then ameliorated with electroacupuncture.[74]

1.4.2. Immunometabolism in IBS

Dysregulated energy metabolism has been observed in a number of functional disorders including cyclical vomiting and chronic fatigue. Less is known about the role this may play in IBS. However , a pilot study of 308 adults with IBS, 102 healthy patients and 36 patients with IBD, assessed two mitochondrial DNA (mtDNA) polymorphisms that were previously implicated in other functional disorders. It found mtDNA polymorphism 16519T was associated with IBS patients, and not IBD, who display maternal inheritance. This suggests mitochondrial dysfunction plays a possible aetiological role in a subgroup of IBS patients. A further study, using a mouse model, found that maternal separation disrupted intestinal mitochondrial structure from childhood to adulthood. This was associated with increased visceral

hypersensitivity, changes in the microbiome and increased intestinal permeability. Currently there is limited further evidence to suggest that dysregulated immunometabolism might play a role in IBS, and there are no treatment approaches currently targeting it.[77,78]

1.5. Natural products and drug discovery

The natural medicinal properties of plants have long been known. In recent history, natural products, and their analogues, have made major contributions to drug discoveries. Natural products are defined as "chemical ingredients synthesized by living organisms via semisynthetic or synthetic methods".[79] There are many examples of well-known natural product discoveries that have high efficacy, such as quinine (*Cinchona* spp.) for malaria treatment and taxol (*Taxus brevifolia*) for cancer. Natural products have already been structurally optimised by nature. They have multiple favourable drug properties including their complex structure, their ability to regulate their own defence systems and an often competitive interaction with other organisms. It is for this reason that they have proven to be so successful in the disciplines of oncology and infectious diseases. Often their safety profile and broad efficacy is known from their use in traditional medicine. Natural products also modulate the gut microbiome. Therefore diseases with known dysbiosis seem a logical target for these therapies. One such product is the commercially available CurQD, a combination of curcumin and QingDai which are herbal compounds previously found to be effective in mild–moderate and moderate–severe ulcerative colitis (UC), respectively.[80,81] A recent placebo-controlled trial found this combination to be effective for inducing response and remission in active UC

patients.[82] Curcumin supplementation has been shown to enrich beneficial gut microbes such as Bifidobacteria, Lactobacilli, and other butyrate-producing bacteria, while reducing potentially pathogenic taxa including Prevotellaceae, Coriobacterales, Enterobacteria, and Enterococci.[83] Similarly QingDai supplementation has been shown to increase the abundance of the beneficial *Bifidobacteria* and reduce the abundance *Bacteriodetes*.[84] It is postulated that some of the beneficial effects observed following CurQD therapy may be attributed to these modulatory effects on the microbiome.[85] Drug discovery through natural products remains in its infancy in IBD.[86,87]

1.5.1. Natural products in IBD and IBS

Complementary and alternative medicine is defined as the use of medical products and practices that are not part of standard medical care. This includes natural products.[8] Between 21-60% of IBD patients are estimated to use these therapies. However, there is limited evidence for their efficacy in this condition. Their use in IBS however is evidence based and recommended by medical professionals. Perhaps the best-known natural product used in IBS is peppermint oil. It is an anti-spasmodic agent, acting through L-menthol's blockade of calcium channels. However the mechanism of action is not fully understood. It has proven efficacy, with network meta-analysis, which included other licenced antispasmodics, ranking it first in terms of efficacy for global symptoms and third for antispasmodic effects.[52,88,89]

Natural products are highly acceptable to patients, who feel therapies are more natural, and less toxic. Use of these therapies may be considered when conventional

therapy has failed or elicited undesirable side effects. However their mechanism of action is often unknown, and they may have unwanted toxic side effects.[89]

Thesis Hypothesis

The exact pathophysiology of both IBD and IBS remains unknown. Untreated disease causes significant morbidity and therapy outcomes remain suboptimal. Therefore, the pathogenesis of both diseases needs to be further elucidated and novel therapeutic strategies are required. UC was selected as the IBD model because inflammation is confined to the colonic mucosa and extends contiguously, enabling more uniform tissue sampling compared to CD.

We undertook our study to define the need for further therapies in IBD, to better understand the pathogenesis of both UC and IBS, to determine how a longstanding therapy (infliximab) works in UC, and to test novel natural extracts for their therapeutic potential in UC and IBS.

It is our hypothesis that metabolism significantly contributes to mucosal inflammation in UC and IBS. Further to this we hypothesise that novel natural plant extracts will elicit an anti-inflammatory response, via their effect on metabolism, in human ex vivo colonic explant tissue taken from patients with UC and IBS. We selected infliximab as a reference therapy, in order to define a metabolic and inflammatory signature against which extract responses are evaluated.

Thesis specific aims

I. To quantify the effectiveness, safety and cost of current treatment strategies used in complex IBD.

II. To investigate whether a specific inflammatory and metabolic profile is associated with UC or IBS.

III. To investigate whether infliximab and/or novel natural plant extracts alter the inflammatory and metabolic profiles in UC and IBS.

Chapter 2: Effectiveness, Safety, and Cost of Combination Advanced Therapies in Inflammatory Bowel Disease

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Alimentary Tract

Effectiveness, safety, and cost of combination advanced therapies in inflammatory bowel disease

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ABSTRACT

Background: A significant proportion of inflammatory bowel disease (IBD) patients fail to respond to advanced therapies. Combining advanced therapies may improve treatment outcome. This study aimed to assess the effectiveness, adverse events, and costs associated with combining advanced therapies in IBD patients.

Methods: Combination advanced therapy was defined as the concurrent use of two biological agents or one biological agent with a small molecule therapy. Clinical data, including disease characteristics, treatment regimens, and adverse events, were collected from electronic patient records. Clinical response rates, biochemical markers, and treatment costs were evaluated.

Results: The study included 109 IBD patients receiving combination advanced therapies from 9 academic centers in Ireland. Corticosteroid-free clinical response rates at 12 weeks and 52 weeks were 39 % and 38 %, respectively. Adverse events occurred in 26 % of therapeutic trials, with disease-related events being the most common. Notably, there were 3 cases of non-melanomatous skin cancer and 10 infectious complications. The annual cost of maintenance therapy for combination advanced therapies ranged from €17,560 to €30,724 per patient.

Conclusion: Combination advanced therapies demonstrated effectiveness and acceptable safety profiles in a cohort of treatment-refractory IBD patients. Further large, prospective trials are required to definitively evaluate the role of combination advanced therapies in IBD.

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2.1. Aims of chapter 2

The overall aim of this chapter is to characterise the gap in treatment efficacy in IBD and highlight the need for novel therapeutic options.

Specific aims include:

1. To characterise the population of IBD patients requiring combination advanced therapies in Ireland.

2. To determine the effectiveness of combination advanced therapies in IBD.

3. To determine the safety of combination advanced therapy use in IBD.

4. To determine the cost of combination advanced therapy use in Ireland.

2.2. Introduction

The introduction of advanced therapies for IBD, including biological and small molecule therapies, over the past two decades has revolutionised treatment approaches and outcomes. Notably, mucosal healing has been associated with higher rates of sustained remission, reduced hospitalisation, and decreased need for colectomy.[90-93] Consequently, a "treat-to-target" approach has been widely adopted whereby mucosal healing has now been recognised as a key desirable endpoint in the latest STRIDE-II guidelines.[29]

Therapy outcomes of advanced therapies appear to remain consistent across trials despite the development of novel agents and new pathophysiological pathways being targeted.[23,56] One suggested approach to overcome this therapeutic plateau is the application of a precision medicine-like strategy for treatment determination. However, until precision medicine techniques are firmly established in the field of IBD, combining advanced therapies has emerged as a more practical approach.[94]

Landmark trials such SONIC and UC-SUCCESS have established the use of combination therapies in IBD (infliximab with azathioprine).[95,96] More recently, focus has shifted towards combining two advanced therapies, such as two biological agents or a biological agent and small molecule therapy, to target the disease through multiple pathophysiological pathways. This method aims to overcome the therapeutic plateau. However, it is essential to consider the associated costs and potential side effects, including immunosuppression, when utilising combination therapy.[97,98] Despite the increasing use of this approach for refractory IBD, data are

few concerning the safety and efficacy of this treatment strategy. It is within this landscape that we undertake this study to determine the efficacy, safety and cost associated with combination advanced therapies.

2.3. Methods

2.3.1. Patient population

A total of 109 patients with IBD who received combination advanced therapies were retrospectively identified from nine academic medical centres participating in the Investigator Network for Inflammatory Bowel Disease Therapy in Ireland (INITIative). All patients included in the study were diagnosed with IBD based on established diagnostic criteria.[5] Patients were included if therapies were commenced for refractory IBD, or for both active IBD and an active immune mediated inflammatory disorder (IMID) or extraintestinal manifestation (EIM) of IBD, or for active IMID or EIM despite quiescent IBD.

Combination advanced therapy was defined as the concurrent administration of two biological agents or one biological agent along with a small molecule therapy. These therapies were either licensed or undergoing clinical trial evaluation for the treatment of IBD. We employed an intention to treat design in this study with all patients receiving combination advanced therapy for any period of time included in analyses. Data collection for this study commenced in October 2022 and concluded in December 2022. Necessary approval was obtained from the research ethics committees at each of the nine participating centres.

2.3.2. Study assessments

Data on included study subjects were collected at each site and stored in an anonymised format. Electronic patient records were reviewed to gather demographic data, and disease and treatment history. Specific details pertaining to each therapeutic trial of advanced combination therapies were documented.

Therapies were classified as per mechanism of action, whereby anti-TNF included infliximab, adalimumab, golimumab and certolizumab; anti-IL-23 included ustekinumab and guselkumab, anti-integrin included vedolizumab; JAK inhibitors included tofacitinib, upadacitinib and filgotinib.

Evaluation of disease activity occurred at baseline, at 12 weeks, and at 52 weeks. A physician global assessment, incorporating both luminal and extraintestinal disease, was conducted by investigators at these time points and graded as per a 5-point Likert scale (1 = complete improvement [defined as resolution of index symptoms to baseline/near-baseline and no treatment escalation], 2 = partial improvement [clinically meaningful improvement without full remission, i.e. a reduction in symptom burden], 3 = situation stable/unchanged [no meaningful change in symptoms], 4 = worsening after initial improvement [initial improvement meeting the "partial" criterion followed by relapse within the assessment window], and 5= worsening [symptom deterioration versus baseline or need for treatment escalation, urgent care/hospitalisation, or new steroid initiation]). Clinical disease activity scores were quantified at these time points, using partial adapted Mayo score for UC and Harvey Bradshaw index for CD (**Table 3** & **Table 4**). The use of systemic steroids was also documented. C-reactive protein (CRP), albumin and faecal calprotectin were recorded at baseline and in the follow-up period. Investigators documented

adverse events occurring in the study follow-up. These were graded according to severity based on the Common Terminology Criteria for Adverse Events (CTCAE) version 5.0 (**Table 5**). The occurrence of serious or opportunistic infections, new or recurrent malignancies, complications of the disease, need for hospitalisation, length of hospital stay, and requirement for admission to the Intensive Care Unit (ICU) were recorded.

	0	1	2	3
Stool Frequency	Normal	1-2 stools/day more than normal	3-4 stools/day more than normal	>4 stools/day more than normal
Rectal Bleeding	None	Visible blood with stool <50% of the time	Visible blood with stool ≥50% of the time	Passing blood alone

Table 3. Partial adapted Mayo score

	0	1	2	3	4
General well-being	Very well	Slightly below average	Poor	Very poor	Terrible
Abdominal pain	None	Mild	Moderate	Severe	
Number of liquid stool per day	None	1 point for each			
Abdominal mass	None	Dubious	Definite	Definite with tenderness	
Complications	None	1 point for each (arthritis, uveitis, erythema nodosum, aphthous ulcers, pyoderma gangrenosum, anal fistula, abscess)			

Table 4. Harvey Bradshaw Index

Grade of adverse event	Description
1	Asymptomatic or mild symptoms; clinical or diagnostic observations only; no intervention indicated
2	Moderate, minimal, local or non-invasive intervention indicated; limiting age-appropriate instrumental activities of daily living (ADL).
3	Severe or medically significant but not immediately life-threatening; hospitalisation or prolongation of hospitalisation indicated; disabling; limiting self-care ADL
4	Life-threatening consequences; urgent intervention indicated
5	Death related to an adverse event

Table 5: Grading of adverse events: Common Terminology Criteria for Adverse Events version 5.0

2.3.3. Study endpoints

The primary endpoint was steroid-free clinical response at weeks 12 and 52. Corticosteroid-free (CSF) clinical response was defined as an investigator assessment of complete or partial response, absence of steroid therapy at the timepoint assessment (12- or 52-weeks), and persistence of combination therapy. If 12-week data were not available, 24-week review data were used to impute status at week 12 i.e. a patient achieving clinical response at week 24 was assumed to have achieved this endpoint at week 12 and vice versa. Patients who discontinued an advanced therapy due to clinical improvement were also considered to be a responder.

Secondary endpoints included CSF clinical and biochemical responses at weeks 12 and 52. This was defined as an investigator assessment of complete or partial response, absence of steroid therapy at the timepoint assessment (12- or 52-weeks), persistence of combination therapy, and normalisation of CRP levels (<5 milligram/litre). Persistence of combination therapy was also assessed as a surrogate marker of treatment success, defined as the duration of time patients remained on combination therapy during follow-up without discontinuation. Other secondary endpoints included changes in biochemical markers (CRP and faecal calprotectin), changes in clinical scores (partial adapted Mayo score for UC and Harvey Bradshaw Index for CD), reasons for combination therapy discontinuation, rates of adverse events, hospitalisation and length of stay, ICU admission, surgery, and occurrence of new or recurrent cancers and infections. A further secondary

endpoint was drug cost analysis. In those that received multiple therapeutic trials of combination regimens, the first therapeutic trial was used for effectiveness analysis.

2.3.4. Definitions for time-based analyses

For patients remaining on combination therapy during the study follow-up, time on therapy was defined as the duration of time (weeks) from therapy initiation to the last follow-up. For patients discontinuing combination therapy, time to discontinuation of therapy was defined as the duration of time (weeks) from the initiation of combination therapies to the discontinuation of one or both advanced therapies.

2.3.5. Cost-effectiveness analysis

The annual cost of maintenance on combination therapy was determined. For all analysis the costing of a standard dosing regimen was used. The cost of optimised dosing schedules was not analysed. Best value biosimilar drug costs were used where applicable. Drugs were priced in subcutaneous format where possible except Infliximab which was priced in intravenous format. For Infliximab a weight of 78.45kg was used to calculate a weight-based dosing of 5mg/kg. This average weight was derived from The National Health Service, Health Service of England, 2021 report.[8] Pricing of infliximab was based on wholesale pricing of the biosimilar inflectra. For oral and subcutaneously administered drugs, pricing was based on nationally negotiated prices in the Republic of Ireland.[99] The cost of induction

regimens and the indirect costs associated with drug administration were not included in analyses. Value Added Tax (VAT) rates differ across the European Union, as such neither were tax costs included in analyses.

2.3.6. Statistical analysis

Baseline demographic and clinical data were presented as medians and interquartile ranges for continuous variables, and as frequencies and percentages for categorical variables. Where data for an individual was missing, summary data was generated using available data. Effectiveness outcomes were assessed via the Mann-Whitney test for non-parametric data and t-test for parametric data. Kaplan–Meier survival curves were constructed for time-based analyses and differences between groups were evaluated using the log-rank test. For calculation of week 12 and 52 CSF clinical and biochemical response rates, percentages were calculated using the number of patients with available data for these endpoints. The $\chi 2$-test was used to assess differences between groups as appropriate. P values < 0.05 were considered significant in analyses. All statistical analysis was done on GraphPad Prism version 9, SPSS (version 9.1.2; IBM, New York, New York, USA) and R Core Team (2023). _R: A Language and Environment for Statistical Computing_. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.

2.4. Results

2.4.1. Baseline demographics

The baseline characteristics of the study cohort (n = 109) are described in **Table 6**. The median age of the patients included in the study was 36 years (IQR 25.3 – 45.0 years); 59.6% were men. 75.2% (n=82) had CD, the remainder had UC. The median disease duration was 10 years (IQR 5 – 18 years). The median number of biologics patients were previously exposed to was 3 (IQR 2-- 3). 50% (n=42) of the CD cohort had undergone IBD related surgery (median number of surgeries (IQR) 0.5 (0-- 2)). No patients in the UC cohort had undergone IBD-related surgery. 2.8% (n=3) of the overall cohort had a previously treated malignancy (one case of melanoma and two of non-melanomatous skin cancer (NMSC)). The case of melanoma, occurring 1.6 years prior to commencing combination advanced therapy, was staged as T1aN0M0 and treated with wide local excision.

A total of 122 therapeutic trials of combination advanced therapies were undertaken in the study cohort (n=109) with 75% of these trials occurring in patients with CD. Nine patients were treated with two different combination therapy regimens sequentially, and two patients were treated with three different regimens sequentially. Overall, 15 different combination regimens were used. The most prescribed regimen was ustekinumab and vedolizumab, comprising 39% (n=42) of initial therapeutic trials. (**Figure 6**) 86% of patients (n=94) commenced therapy for refractory IBD, 8% (n=9) for both active IBD and an IMID or EIM of IBD, and 6% (n=6) for active IMID or EIM despite quiescent IBD. Five patients were given

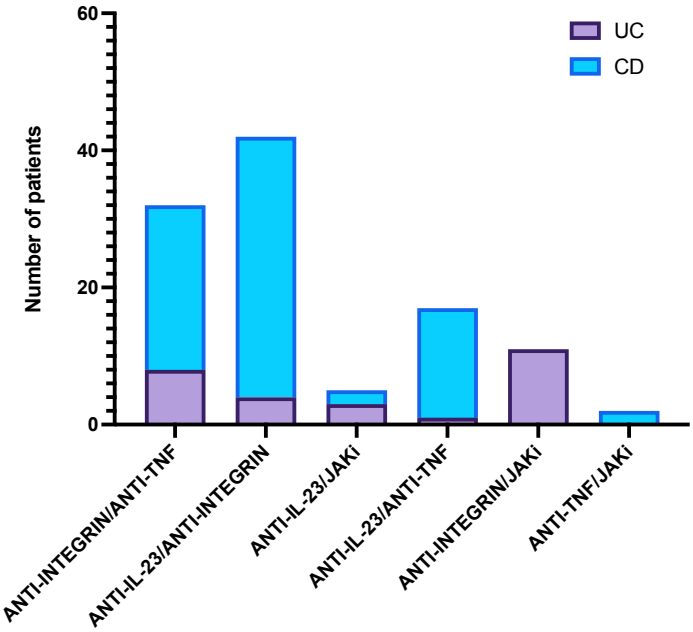
concurrent induction of two advanced therapies. All of these five patients had refractory luminal IBD. One patient had a diagnosis of UC, with the remaining five having CD. Two of these patients were on oral steroids at initiation, one of whom was also on exclusive enteral nutrition.

Data on the use of PJP prophylaxis was available for 93% (n=101) of patients during their first therapeutic trial. Among these patients, 27% (n=27) commenced dual advanced therapies with PJP prophylaxis. 82% (n=22) of those who received PJP prophylaxis were concurrently on systemic corticosteroids. Data on dose intensification was available for 77% (n=84) of patients during their first therapeutic trial. 54% (n=45) of these patients underwent dose intensification during treatment.

Baseline Characteristic		All	UC		CD
Number of patients (%)		109	27 (24.8)		82 (75.2)
Therapeutic trials		122	31 (25.4)		91 (74.6)
Male sex, n (%)		65 (59.6)	16 (59.3)		49 (59.8)
Age [years], median, (IQR)		36 (25.25-45)	35 (24-46)		36.5 (26-45)
Age at diagnosis of IBD [years], median (IQR)		22 (16-33)	23.5 (15-42.5)		21 (16-31.5)
Disease duration [years], median (IQR)		10 (5-18)	7 (4-12)		11 (6.5-19)
Smoking status, n (%)	Never	73 (67)	20 (74.1)		53 (64.6)
	Current	9 (8.3)	1 (3.7)		8 (9.8)
	Ex-smoker	17 (15.6)	2 (7.4)		15 (18.3)
Montreal Classification, n (%)	E1: Ulcerative proctitis		1 (3.7)	L1: Ileal	7 (8.5)
	E2: Left-sided (distal to splenic flexure)		9 (33.3)	L2: Colonic	19 (23.2)
	E3: Extensive (proximal to splenic flexure)		15 (55.6)	L3: Ileocolonic	46 (56.1)
				L3+L4: Ileocolonic + upper GI	8 (9.8)
				Unknown	1 (1.2)
				B1: Non-stricturing, non-penetrating	28 (34.1)
				B2: Stricturing	25 (30.5)
				B3: Penetrating	26 (31.7)
				P: Perianal	34 (41.5)
					*Isolated perianal disease n=1
Extra-intestinal manifestation of IBD, n (%)		31 (28.4)	4 (14.8)		27 (33)
- PSC, n (%)		7 (6.4)	3 (11.1)		4 (4.9)
Associated immune mediated disease, n (%)		13 (11.9)	0		13 (15.9)
Prior IBD surgery, n (%)		41 (37.6)	0		41 (50)
Number of surgeries, median (IQR)					0.5 (0-2)
Number of prior biologics, median (IQR)		3 (2 – 3)	3 (2-3)		3 (2-3)
Prior immunomodulator use, n (%)		85 (78)	17 (63)		68 (83)
Previous malignancy, n (%)		3 (2.8)	0		3 (3.7)
					n=1 melanoma
					n=2 NMSC (BCC)

Table 6: Baseline characteristics of the study cohort at the time of combination advanced therapy initiation. CD, Crohn‘s disease; interquartile range; NMSC, non-melanomatous skin cancer; PSC, primary sclerosing cholangitis; UC, ulcerative colitis.

A.



B.

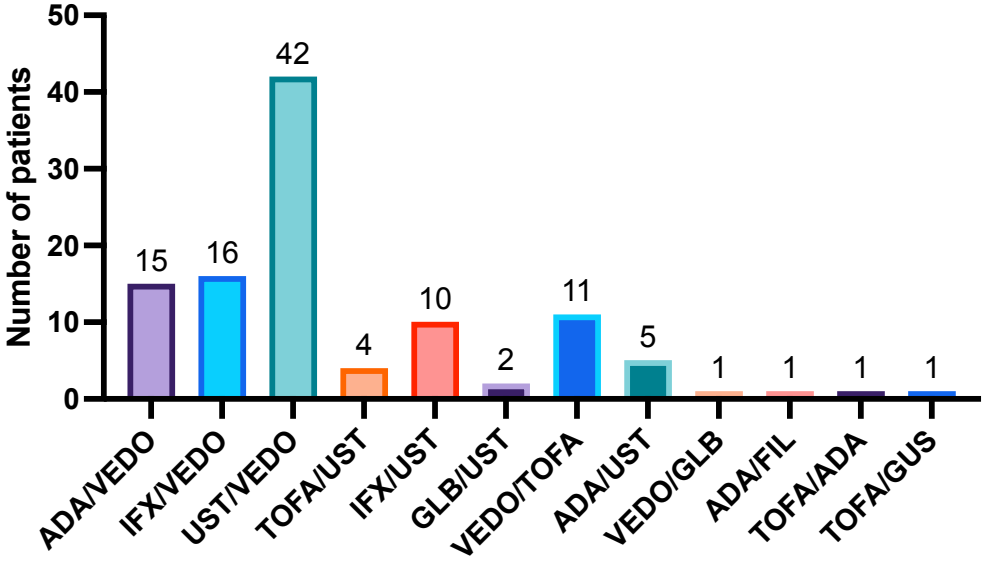


Figure 6: Combination advanced therapy regimens used in first therapeutic trial in study cohort. A. Overall, six different combinations of class of therapy were trialled, with blue representing CD and purple UC. B. Names of drugs in combination regimen used during the first therapeutic trial. The most commonly

prescribed regimen was ustekinumab and vedolizumab. CD, Crohn's Disease; IL, interleukin; JAK, Janus kinase; TNF, tumour necrosis factor; UC, ulcerative colitis.

2.4.2. Combination therapy outcomes

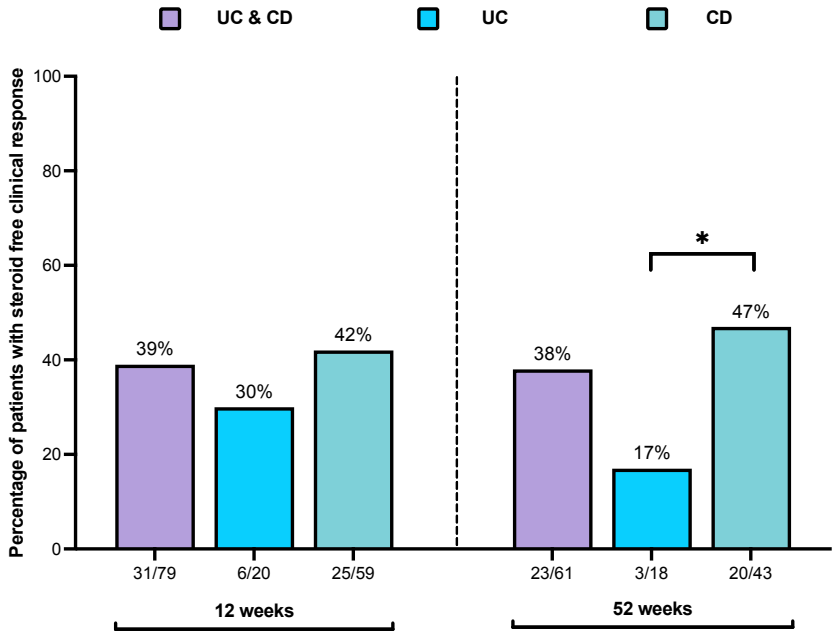
79 and 61 patients had available data for assessment of week 12 and 52 CSF clinical response respectively. 39% and 38% of patients achieved week 12 and 52 CSF clinical response respectively. 29% and 22% achieved week 12 and 52 CSF clinical and biochemical response respectively (**Figure 7**). Of the 65 patients who had an abnormal CRP at baseline (CRP > 5 mg/L) only 14% (7/49) had CSF clinical and biochemical response at week 12 and 23% (3/30) at week 52. At week 52, a greater proportion of patients with CD (47%) compared with UC (17%) achieved week 52 CSF clinical response, p=0.042. There was a non-statistical trend towards higher response rates in CD than UC for all other assessed treatment outcome endpoints.

Combination of anti-TNF and anti-IL23 therapies was the most effective regimen at 12 weeks with 56% of patients achieving CSF clinical response and 44% achieving CSF clinical and biochemical response. At 52 weeks a combination of anti-IL23 and JAK inhibitor was the most effective, with 67% of patients achieving CSF clinical response and 33% achieving CSF clinical and biochemical response. However numbers in this subgroup were small (n=3) [**Table 7**].

	12 weeks				52 weeks			
	CSF clinical response		CSF clinical and biochemical response		CSF clinical response		CSF clinical and biochemical response	
Combination	Number with available data	% responders	Number with available data	% responders	Number with available data	% responders	Number with available data	% responders
ANTI-IL23/ANTI-INTEGRIN	34	38.2	34	11.8	20	35	19	15.8
ANTI-INTEGRIN/ANTI-TNF	26	38.5	26	30	22	45.5	22	27.3
ANTI-TNF/ANTI-IL23	9	55.5	9	44.4	8	37.5	8	25
ANTI-INTEGRIN/JAKi	9	33.3	9	11.1	10	60	10	30
ANTI-IL23/JAKi	3	33.3	3	33.3	3	66.7	3	33.3
ANTI-TNF/JAKi	2	0	2	0				

Table 7: Response rates for different combination regimens used. CSF, corticosteroid-free; IL, interleukin; JAK, Janus kinase; TNF, tumour necrosis factor.

A.



B.

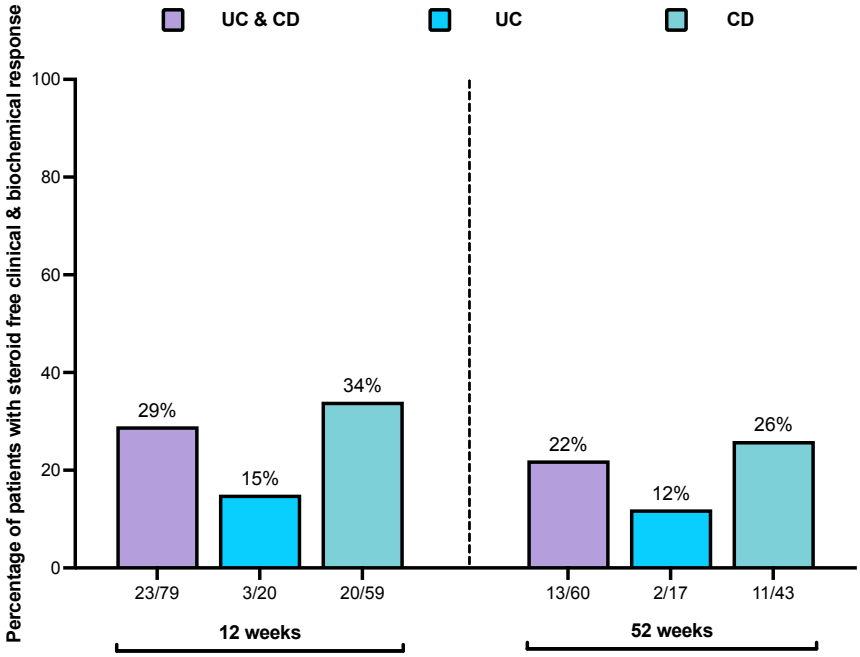


Figure 7: Clinical response to combination therapy at weeks 12 and 52 in the overall study cohort and by disease subtype. The overall cohort is represented by the colour purple, blue represents UC, and green represents CD. CD, Crohn's Disease; UC, ulcerative colitis.

A.) Percentage of patients with CSF clinical response. At week 52 a greater proportion of CD patients achieved clinical response than UC patients.

B.) Percentage of patients with CSF clinical & biochemical response. There was no difference in proportions achieving clinical and biochemical response across the UC and CD cohorts.

2.4.3. Inflammatory biomarker and disease activity score response to combination advanced therapies

For patients receiving combination therapy, median CRP levels reduced from a baseline of 6 mg/L [IQR 2.35–- 19.8 mg/L] to 2 mg/mL [IQR 1 – 9 mg/L] at 52 weeks of therapy (p=0.0001). Similarly faecal calprotectin levels reduced from baseline to week 52 with median concentration reducing from 899 µg/mL [IQR 337.6 – 1808 µg/mL] to 122 µg/mL [IQR 22 – 576 µg/mL] (p=0.0001) (**Figure 8 & Table 8**).

There was a significant reduction in clinical disease activity scores on combination therapy. In CD, the median Harvey Bradshaw Index reduced from 8 [IQR 5.5–- 11] at baseline to 3 [IQR 1–- 4] at week 52, p<0.0001. In UC, the median partial adapted Mayo score reduced from 7 [IQR 4–- 8] at baseline to 2 [IQR 1–- 3] at week 52, p<0.0001. (**Figure 8 & Table 8**)

	Baseline	3 months	6 months	1 year	2 years	P value
Number of patients at time point	109	84	70	45	24	
CRP (mg/L), median (IQR)	n=105	n=67	n=53	n=38	n=16	
	6 (2.35 – 19.8)	3.3 (1.2 - 8.6)	3 (1 - 8.8)	2 (1 - 9)	2 (1 - 5.5)	<0.0001
Calprotectin (µg/g), median (IQR)	n=67	n=28	n=23	n=20	n=10	
	899 (337.6 - 1808)	622 (203 - 1659)	568 (113 - 1595)	122 (22 - 576)	67 (28.25 – 248)	0.0002

Table 8. Inflammatory biomarkers at baseline, 3 month, 6 month, 1 year and 2 year time points. CRP, C-reactive protein; IQR, interquartile range.

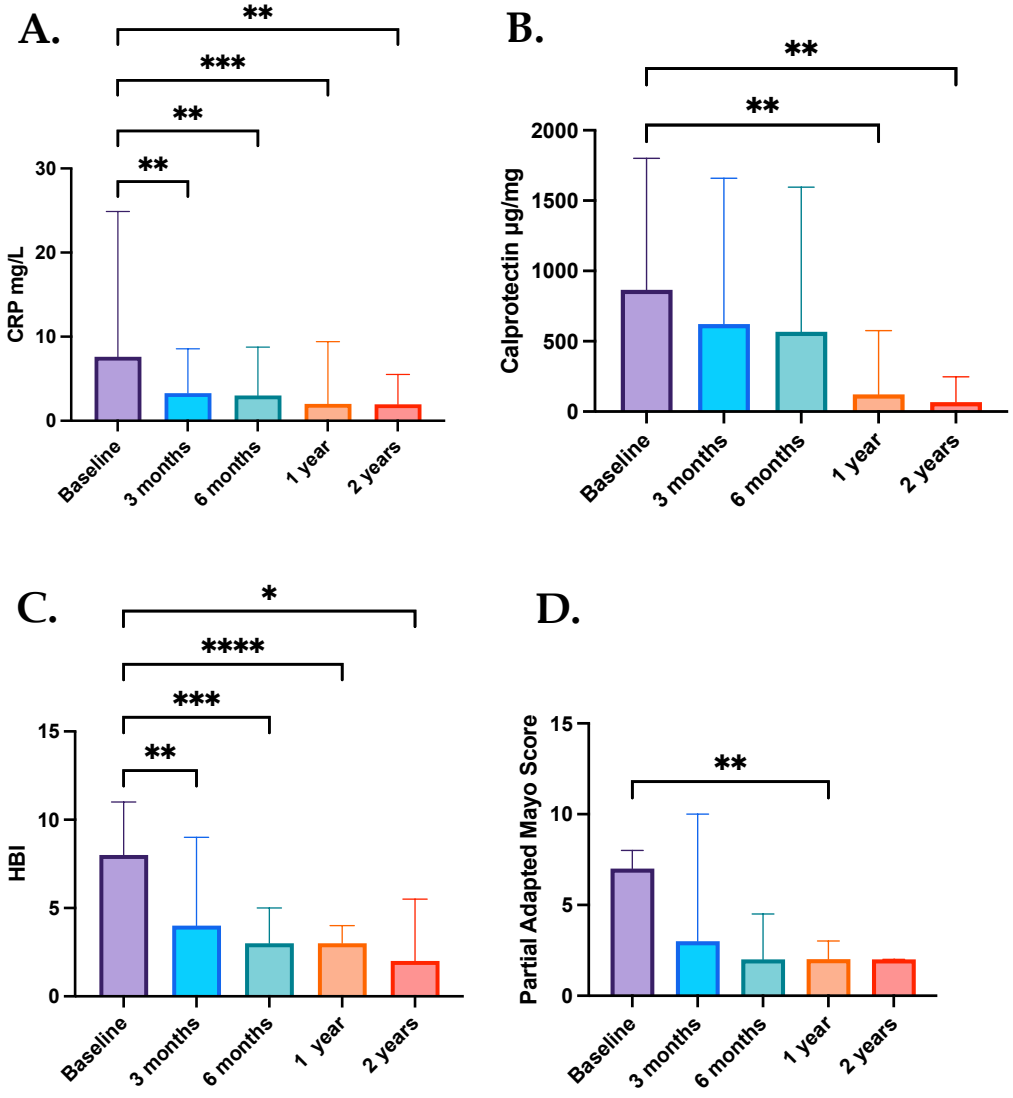


Figure 8: Inflammatory biomarker and disease activity score response to combination advanced therapies. A.) Median and IQR CRP levels B.) Median and IQR faecal calprotectin levels C.) Median and IQR Harvey Bradshaw index for CD patients D.) Median and IQR partial adapted Mayo score for UC patients. CD, Crohn's Disease; CRP, C-reactive protein; HBI, Harvey Bradshaw Index; IQR, interquartile range; UC, ulcerative colitis.

2.4.4. Persistence of combination therapy during follow-up

28 of 109 (26%) patients discontinued combination therapy during study follow-up. The median duration of follow-up was 39 weeks [IQR 14–- 79.5]. Reasons for discontinuation of combination therapy included; treatment success (n=2), patient preference (n=2), adverse reaction (n=2, infusion reaction and recurrent infection), and ongoing luminal disease activity (n=22). The mean [95% confidence interval (CI)] time to first discontinuation of first therapeutic trial of combination therapy was 154.9 weeks (129.6 – 180.2 weeks) (**Figure 9**). There was no difference in time to discontinuation of combination therapy comparing patients with CD [mean (95% CI), 170.8 weeks (143.1 – 198.6 weeks)] versus UC [91 weeks (63.6 – 118.3 weeks)], p=0.072. There was no statistical difference in drug persistence between different combination regimens when grouped by class (p = 0.071). 11 patients underwent a 2nd therapeutic trial of a different combination regimen, the mean time to discontinuation of therapy was 46.9 weeks (21.6 – 72.2 weeks). Two patients underwent a 3rd therapeutic trial of a different combination regimen. Both patients remained on these therapies at time of last follow-up, with time on therapy ranging from 3 to 24 weeks.

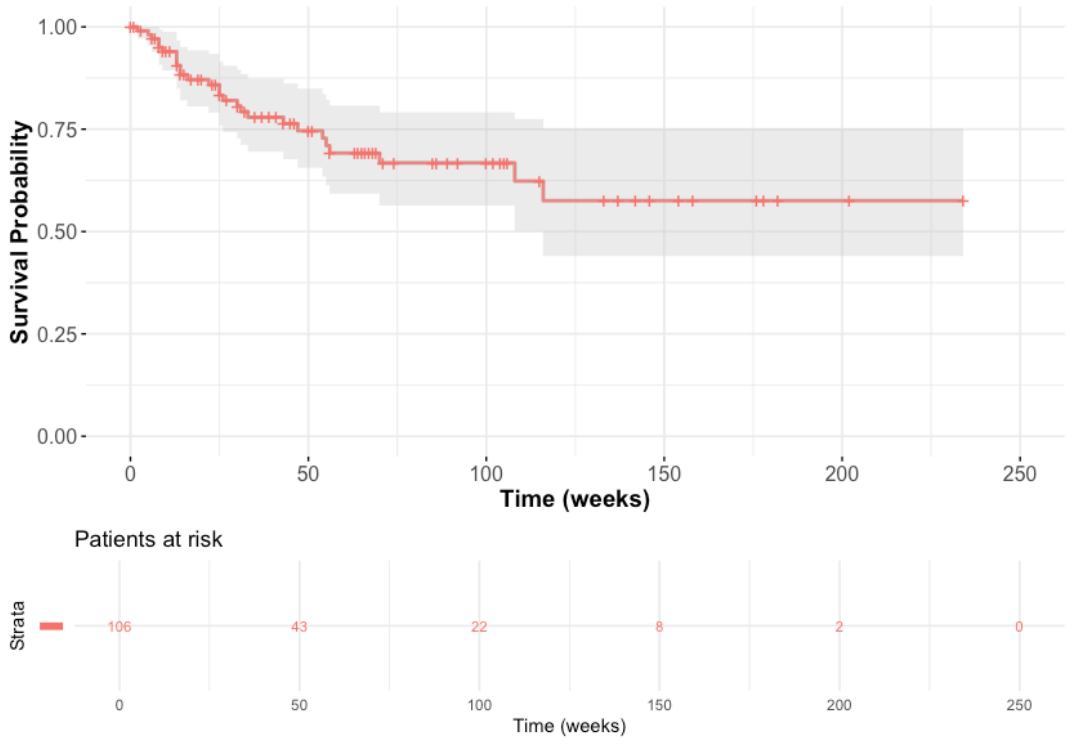


Figure 9: Combination therapy persistence in the study cohort. 28 patients discontinued combination therapy during study follow-up. The mean (95% confidence interval) time to combination therapy discontinuation was 154.9 weeks (129.6 – 180.2).

2.4.5. Safety and adverse events

Adverse events occurred in 26% (n=32) of therapeutic trials (**Table 9**). Most adverse events were graded as moderate. Adverse events relating to active IBD were the most common (n=16). 16 patients underwent IBD-related surgery, including one with a bowel perforation. A higher rate of bowel resection was noted numerically in the UC cohort versus the CD cohort (five patients versus four patients); however, there was a higher volume of missing data in the CD cohort (14 patients versus two patients respectively).

Ten infective complications were recorded with only two serious infections (one patient developed severe *C. Difficile* infection and another a tubo-ovarian abscess). The patient who developed *C. Difficile* was on ustekinumab and vedolizumab. No other opportunistic infections were recorded. There were two cases of venous thromboembolism, neither occurred in the setting of JAK inhibitor use. There were three cases of NMSC, two of the three cases had previously been treated for a NMSC. None of the patients who developed a NMSC received an immunomodulator whilst on combination advanced therapy.

28 patients were admitted to hospital, with 68% having IBD-related hospitalisation. The median length of stay was seven days (IQR, 2 – 14 days). There were no deaths or admissions to the intensive care unit.

	Number of adverse events
Disease related	16
Infective	10
Enteric infections - *Clostridium Difficile*	2 - 1
Respiratory infections	5
Tubo-ovarian abscess	1
Perianal sepsis	2
Venous Thromboembolism - Pulmonary embolism	2 - 2
Drug reaction	2
Infusion reaction	1
Injection site reaction	1
New or recurrent malignancies	3
NMSC	3
Other	5
Myalgia	1
Sacral insufficiency fracture	1
Haemoptysis	1
Hyponatraemia	1
Chest pain	1

Table 9: Adverse events in study cohort. NMSC, non-melanomatous skin cancer.

2.4.6. Cost-effectiveness of combination therapy

Assuming all patients remained on therapy for one year at the lowest licenced dose, the annual cost of maintenance therapy of the first therapeutic trial of combination therapy in our cohort of 109 patients was €2,864,792. The average cost of treatment per patient was €24,154 per annum (range €17,560 - €30,724). vedolizumab and ustekinumab was the most costly combination regimen used (€30,723.60/annum/patient). All prices are exclusive of VAT (**Table 10**).

Combination Regimen	Cost of maintenance therapy per annum
Adalimumab & Vedolizumab	€20,766.96
Infliximab & Vedolizumab	€23,079.36
Ustekinumab & Vedolizumab	€30,723.60
Tofacitinib & Ustekinumab	€27,516.84
Infliximab & Ustekinumab	€26,851.44
Golimumab & Ustekinumab	€28,798.08
Vedolizumab & Tofacitinib	€23,744.76
Adalimumab & Ustekinumab	€24,539.04
Vedolizumab & Golimumab	€25,026.00
Adalimumab & Filgotinib	€18,182.67
Tofacitinib & Adalimumab	€17,560.20
Tofacitinib & Guselkumab	€23,061.18

Table 10: Cost analysis of maintenance therapy for one year, not on optimised dose. Same comment

2.5. Discussion

Despite the considerable progress made in the field of IBD therapeutics, there are still challenges in achieving optimal response rates in a cohort of patients, where there appears to be a plateau of drug efficacy. Combining two advanced therapies aims to overcome this by targeting different inflammatory pathways. This has become an increasingly common treatment strategy, particularly in cases of refractory disease. However, there is limited data available regarding the outcomes and potential risks associated with this approach. In this study, we aimed to provide insights into the clinical outcomes, adverse events and cost implications of combination advanced therapies in the treatment of IBD patients.

In concordance with previous reports evaluating combination advanced therapies for IBD, the patients included in this study had severe IBD phenotypes.[1,100,101]. The group also had a number of risk factors for treatment failure including significant prior biologic exposure and long disease duration. The IBD patients evaluated in this study often fail to meet clinical trial inclusion criteria and therefore real-world data on therapy outcomes provide important information for clinicians.[102-105]

Previous studies evaluating the outcome of combination biologic therapy in IBD have generally been retrospective with significant heterogeneity in the reporting of treatment outcomes. Our study used a 5 point Likert scale to assess clinical response which was similar to the treatment outcome endpoint definition of a European retrospective observational study from the COMBIO group.[100] In this study, complete or partial response was observed in 70% of patients receiving therapy for

active IBD. Two meta-analyses have evaluated the outcome of combination biologic therapy in IBD. A 2022 meta-analysis by *Ahmed et al.* reported a pooled clinical remission rate of 59% (95% CI, 42%-74%), and endoscopic remission rate of 34% (95% CI, 23%-46%) on combination therapy. Further to this *Alayo et al.* reported pooled response rates for individual combination regimens in their meta-analysis. Our study primarily focused on overall treatment outcomes across all combination regimens. The data from our study is largely in concordance with the pooled clinical remission rates reported by *Ahmed et al*. We demonstrated week 12 and 52 CSF clinical response rates of 40% in our cohort. Response to combination biologic therapy appeared to be durable with 74% of patients remaining on therapy at the end of study follow-up. In those patients who remained on therapy, there was a favourable inflammatory biomarker response with CRP and faecal calprotectin reducing from baseline over the study follow-up period following the introduction of therapy.[1,101]

Higher rates of clinical response were observed in patients with CD compared to those with UC at 52 weeks. However interestingly, adding the composite endpoint of clinical and biochemical response produced a marked drop in week-52 response, particularly in CD. This likely reflects the greater stringency of the combined endpoint. There was also a non-statistical trend towards higher rates of response and drug persistence in the CD cohort. Increased biological therapy persistence and efficacy rates in CD versus UC have been reported previously. In a recent Danish nationwide cohort study, persistence of therapy was used as a proxy for treatment response. Treatment persistence was lower in the UC compared with the CD cohort with persistence rates at 1 year 44% in UC and 56% in the CD group. At 3 years this reduced to 17% in UC and 34% in CD patients.[106] A further Danish study involving

a retrospective review of outcomes of all bio-naïve IBD patients who initiated biological therapy in one centre over a 10-year period, found that first biological therapy was discontinued after a median of 5 months (IQR 2–13) for UC versus 10 months (IQR 4–20) for CD patients. Primary non-response rates were also numerically higher in the UC group. Notably the 5 year surgery rate was higher in the UC compared with the CD cohort (26.6% versus 20.4%)[107] The findings in our study are in keeping with the literature demonstrating decreased combination therapy persistence in UC compared with CD patients and possible higher rates of colectomy. The reasons are likely multifactorial and include heterogeneity in therapy response between the two disease phenotypes, differing approaches to treatment of each disease subtype and a likely lower threshold for surgical referral in the UC cohort.

In our study, anti-TNF/anti-IL23 combination therapy was the most effective regimen following 12 weeks of therapy with greater than 55% of patients demonstrating clinical response. Pooled data from a 2022 meta-analysis from Alayo et al. reported higher response rates of 91% [95% CI, 64.1-100; 5 studies] for this combination regimen[101]. For studies included in this meta-analysis, there was substantial heterogeneity in the definition of response, the time point of response assessment and a short duration of follow-up. In our study 15 different combination regimens were used across all therapeutic trials. Patient numbers were small in certain therapeutic regimen groups and there was significant heterogeneity in baseline disease characteristics. For these reasons we elected to compare treatment outcomes between combination therapy regimens by drug class rather than agent specific comparisons. Whilst we compare the effectiveness of different combination regimes, given the limitations mentioned above, these results should be interpreted

with caution. Larger randomised controlled trials are currently underway investigating the use of combination regimens as induction and maintenance therapies in UC and CD. These are anticipated to generate high quality data supporting the use of combination advanced therapies in treatment refractory IBD.

(https://classic.clinicaltrials.gov/ct2/show/NCT05242484 & https://classic.clinicaltrials.gov/ct2/show/NCT05242471).[3,108,109]

Ustekinumab and vedolizumab were the most frequently prescribed combination regimen in our cohort. Although the rationale for combination selection was not documented, both agents have favourable safety profiles in IBD, suggesting that safety considerations may have influenced prescribing decisions.[110] A favourable safety profile of combination advanced therapies was demonstrated in our study. No new safety signals were observed. Adverse events were observed in 26% of all therapeutic trials. However 50% of all adverse events were disease related, with 75% of this group requiring hospitalisation due to IBD disease activity. This finding reflects the severity of disease in this study cohort and should be considered as an absence of response to treatment rather than a complication of combination therapy.

The majority of all adverse events were graded as moderate. Three cancers were reported in the study follow-up period all of which were NMSC. Prospective data regarding safety of combination biologic use in IBD are limited. Given the paucity of prospective data pertaining to the safety of combination advanced therapies in IBD, information can be extrapolated from rheumatological literature. A 2019 meta-analysis of controlled studies by Boleto et al. of patients with rheumatoid arthritis on combination advanced therapies suggested an increased incidence of adverse

events in the combination group (15%) versus the monotherapy group (6%), OR 2.51 (95% CI 1.29–4.89).[111] It is however important to note that there are considerable differences in the patient profile and the trial included biological therapies not used in IBD which have differing side effect profiles [112]. The two aforementioned meta-analyses, assessing combination advanced therapies in IBD do not report an increase in adverse events comparing combination with monotherapy groups. [1,101]

Biological and small molecule therapies are the most effective treatment for moderate to severe IBD and have improved patient outcomes significantly. However these therapies are costly and account for up to a third of annual disease-attributable costs[113-115]. Whilst our study shows that in real world clinical practice combining these therapies can be an effective treatment for those with the most treatment resistant disease subtypes, it also shows that this approach is costly, with maintenance therapy costing on average €24,154 per patient annually. This costing model provides a conservative estimate of the actual cost incurred from combination advanced therapies. The model did not account for patients requiring dose intensification, nor did it account for indirect costs associated with therapy administration or after tax costs.

The annual cost of combination advanced therapies, ranging from €17,560 to €30,723 in our cohort, should be interpreted in the broader context of IBD care costs. A single biosimilar therapy such as infliximab or adalimumab typically costs in excess of €7,000 per patient annually. However, direct health care expenditures for IBD extend beyond medications, with mean annual per-patient costs estimated at $9,000 (Canadian Dollars) for ulcerative colitis and $12,000 (Canadian Dollars) for Crohn's

disease in high-income settings.[116] Importantly, indirect costs, including absenteeism, presenteeism, and reduced productivity, are substantial. In a nationwide Swedish cohort, the annual societal costs of CD and UC were estimated at $22,813 (US Dollars) and $14,136 (US Dollars) per working-age patient, respectively, encompassing inpatient and hospital-based outpatient care, medications, surgery, and productivity loss. Notably, productivity losses accounted for the majority of costs (56% in CD and 59% in UC), primarily through sick leave and disability pensions.[117] Furthermore, in the pre-biologic era, hospitalisations and surgeries accounted for the majority of direct IBD-related expenditure; over 60% in the United States (up to $25 billion [US Dollars] annually) and 53% in a European inception cohort.[116] Thus, while the upfront drug costs of combination therapy are considerable, these expenses may be offset if such strategies reduce hospitalisations, surgeries, or productivity losses, underscoring the importance of evaluating costs from both health system and societal perspectives. However, given the modest treatment benefits and significant drug costs this study affirms current practice, where combination advanced therapy is reserved for treatment refractory IBD patients.

We acknowledge the limitations of this retrospective study, including the lack of prespecified assessments of clinical, biochemical, and endoscopic activity at specific time-points. Assessments relied on routine clinical care, resulting in some missing outcome data at 12 and 52 weeks, most particularly biochemical biomarker data. Endoscopy and imaging were not protocolised in this real-world cohort, repeat assessments within the response window were sparse and indication-driven, leading to substantial, likely non-random missingness; therefore, we excluded this

data from analyses. Additionally, the multicentre design introduced therapeutic heterogeneity, and the study may have been subject to referral centre bias. Unfortunately, estimation of the cost of dose escalated therapy was not possible due to the lack of recording of patient weights and dose escalation received. Despite these weaknesses, this report stands as one of the largest real-world studies of combination therapy outcomes in IBD. The multicentre design provides valuable insights into trends of prescribing of advanced therapies across different units. Notably, it is the first study in this field to assess the cost implications of combining advanced therapies, providing relevant information amid the increasing use of these agents in combination regimens.

2.6. Conclusion

In conclusion, this study offers insights into the effectiveness, safety, and cost of combination advanced therapies for IBD management. While these therapies show moderate effectiveness and acceptable safety in refractory IBD, their costs are considerable. Despite two pathophysiological pathways being targeted, the therapeutic ceiling of treatment efficacy has not been breached, highlighting the need for novel therapies in this area.

Head-to-head randomised controlled trials are required to definitively establish the efficacy and safety of combination advanced therapies in IBD, comparing monotherapy with dual advanced therapies. Future studies should select a homogenous patient group, with similar IBD phenotypes and treatment history. Consideration should be given to include a less treatment refractory cohort, with

fewer previous treatment failures, and therefore an increased likelihood of therapy success.

2.7. Summary of findings of chapter 2

This chapter adds to the evidence supporting the use of combination advanced therapies in treatment refractory IBD. It describes the effectiveness, safety, and cost of combination advanced therapies in patients with IBD.

A total of 109 IBD patients from nine centres were included, receiving combination therapies involving two biological agents or one biological agent with a small molecule therapy. The primary endpoint was corticosteroid-free clinical response (CSF) at 12 and 52 weeks. At week 12, 39% of patients achieved CSF clinical response, while 38% achieved this at week 52. Clinical and biochemical response rates were 29% at week 12 and 22% at week 52.

Adverse events occurred in 26% of therapeutic trials, with most events being moderate. The most common were disease-related. Infections (10 cases, with 2 serious) and 3 cases of non-melanomatous skin cancer (NMSC) were reported. No new safety signals were identified, and no deaths or ICU admissions occurred. The average annual cost of maintenance therapy was €24,154 per patient, with costs ranging from €17,560 to €30,724, depending on the regimen.

The chapter concludes that combination advanced therapies provide moderate effectiveness and an acceptable safety profile for refractory IBD patients but are associated with considerable cost. This chapter highlights that within this treatment refractory cohort of IBD patients, novel therapies are required to break the therapeutic ceiling of treatment efficacy that exists in IBD.

Chapter 3: Profiling Inflammation and Metabolism in Ulcerative Colitis and Irritable Bowel Syndrome

3.1. Aims of chapter 3

The overall aim of this chapter is to characterise the inflammatory and metabolic profiles of healthy controls (HC), UC patients, and IBS patients.

Specific aims include:

1. To investigate whether there are differences in secretion of ten inflammatory proteins (IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α) from colonic explants derived from HC, UC patients, and IBS patients.

2. To investigate whether there are differences in colonic explant cellular metabolism in HC, UC, or IBS, using Seahorse Extracellular Flux analyser.

3. To investigate whether there are differences in inflammatory and metabolism profiles in HC, UC patients, or IBS patients, using Principal Component Analysis.

4. To investigate whether inflammatory protein secretion correlates with markers of cellular metabolism.

5. To investigate whether inflammatory protein secretion and metabolism markers correlate with baseline clinical characteristics or disease outcomes in UC.

3.2. Introduction

Digestive diseases are very common globally. In Europe alone, over 332 million people are estimated to be living with a digestive disease.[118] Inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) are digestive diseases that cause considerable impact on affected individuals' quality of life, and indeed on society as a result of the economic cost of care and missed work days. While traditionally IBD and IBS were considered to represent opposite ends of the spectrum of intestinal disorders (inflammatory versus functional), research more recently has pointed to some overlapping pathophysiology of disease. Both involve dysregulated genetic, microbial, epithelial and immunologic factors. Great advances have been made in elucidating this pathophysiology, leading to more targeted treatment options; however, there remains an unmet need for improved diagnosis and management of both conditions.[53,119]

Ulcerative colitis (UC) is a form of IBD characterised by chronic inflammation of the colon, extending proximally in a continuous manner from the rectum. The prevalence is estimated to be 5 million cases worldwide, with an increasing incidence. The exact aetiology of UC is unknown. Current evidence suggests that UC occurs in individuals with a genetic predisposition following exposure to environmental triggers. This causes colitis through a complex interplay between a defective epithelial gut barrier, changes in the intestinal microbiome and a dysregulated immune response.

Despite a multitude of new therapies coming to market in recent years, there remains a therapeutic ceiling of treatment efficacy, with 10-20% of individuals requiring surgical management for medically refractory disease. A considerable proportion of UC patients in endoscopic remission have ongoing symptoms, perhaps suggesting an overlay of functional disease; changes in intestinal permeability have been noted in this setting, however. This underscores the need for further exploration into the local intestinal changes associated with both inactive and active UC.[21,120]

IBS is a common disorder of the gut-brain axis, affecting roughly 1 in 10 individuals globally. The aetiology of IBS is unknown and appears to differ depending on geographic location. Genetics, diet, alterations in the gut microbiome, gastrointestinal infection and psychological factors have all been implicated in disrupting the gut-brain axis. Treatments include dietary, psychotherapy, pharmacotherapy and microbial interventions. These have varying degrees of success, with most patients failing first line therapies.[53,56]

Given the gap in efficacy of treatment in both UC and IBS, novel therapeutic targets are required. Immunometabolism, which involves altering cellular metabolism to influence inflammatory cells towards an anti-inflammatory profile, has emerged as one such target in IBD. There remains a knowledge gap in understanding the underlying interaction between inflammation and metabolism within IBD, and little is known about this discipline in IBS.[121] It is within this framework that we investigate inflammatory and metabolism profiles in HC, IBS patients and active and inactive UC patients using ex vivo human colonic explants.

3.3. Methods

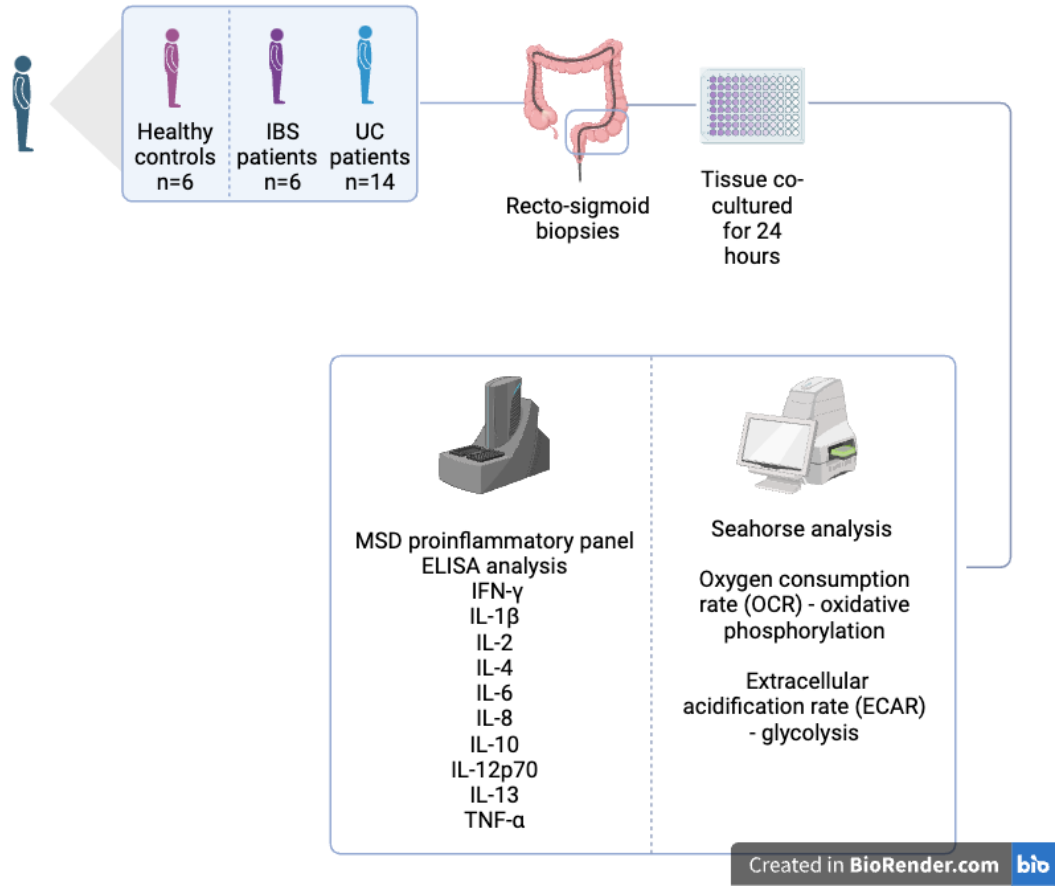


Figure 10: Experimental design characterising the inflammatory and metabolic profiles in healthy controls, ulcerative colitis, and irritable bowel syndrome. HC, UC patients and IBS patients were recruited at endoscopy. Rectosigmoid biopsies were collected. Colonic tissue explants were generated. Seahorse Xfe24 analyser was used to generate metabolism profiles. MSD pro-inflammatory panel undergoing ELISA analysis was used to generate inflammatory profiles. Inflammatory markers included IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α. ECAR, extracellular acidification rate; ELISA, enzyme-linked immunosorbent assay; HC, healthy controls; IL, interleukin; IFN, interferon; IBS, irritable bowel syndrome; OCR, oxygen consumption rate; TNF, tumour necrosis factor; UC, ulcerative colitis.

3.3.1. Study population

A cohort was prospectively recruited at St James's Hospital, Dublin, following approval by The St. James' Hospital / Tallaght University Hospital Joint Research Ethics Committee. Patients were recruited in four cohorts: healthy controls (HC), those with IBS, those with UC in remission, and those with active UC. Baseline demographics and information on medication exposure at the time of endoscopy were collected for each subject. Baseline CRP and faecal calprotectin were recorded where available. An endoscopic Mayo sub-score was documented for each included UC subject.

HC were individuals without gastrointestinal symptoms, who underwent endoscopy for reasons such as family history or polyp surveillance and who were confirmed to have no evidence of colitis on colonoscopy. The diagnosis of IBS was established using recognised clinical, endoscopic, and histological criteria.[52] Patients with a preexisting IBS diagnosis who experienced increased symptoms but had a normal colonoscopy were included in the IBS cohort. The diagnosis of UC was made using established clinical, endoscopic and histological criteria.[5,122] UC patients undergoing endoscopic assessment for evaluation of disease activity, treatment response or for surveillance were included. UC in remission was defined as an endoscopic Mayo score of 0 or 1. Active UC was defined as an endoscopic Mayo score of 2 or 3. (**Table 11**)

Table 11.

Endoscopic Mayo subscore grade	Description
0	Normal mucosa
1	Erythema, decreased vascular pattern, mild friability
2	Marked erythema, absent vascular pattern, friability, erosions
3	Spontaneous bleeding, ulceration

Endoscopic Mayo score. Ulcerative colitis mucosal changes are graded 0 to 3 as per the descriptions listed in the table. Endoscopic Mayo score of 0 to 1 is defined as UC in remission. Endoscopic Mayo score of 2 to 3 is defined as active UC. UC, ulcerative colitis.

3.3.2. Study endpoint definitions

The primary study endpoint was to determine if the four cohorts HC, IBS, UC in remission, and active UC, had different inflammatory and metabolic profiles. Secondary endpoints included determining whether energy metabolism markers were associated with different inflammatory protein responses and clinical variables including disease duration, faecal calprotectin, C-reactive protein (CRP) and disease progression in the UC cohorts. UC disease progression was defined as the requirement for UC-related hospitalisation, colectomy, corticosteroids or new advanced therapy at the time of the study endoscopic assessment or at a time subsequent to this assessment. Therapy changes prior to endoscopy, advanced therapy dose escalation, and 5-ASA therapy changes were not considered in this definition. UC advanced therapy was defined as a biological or small molecule agent.

3.3.3. Ex vivo ulcerative colitis tissue explant culture

All patients undergoing colonoscopy had study-related endoscopic biopsies taken. These biopsies were collected from the sigmoid colon approximately 20 cm from the anorectal margin. The most severely inflamed area was targeted in the UC cohorts. The endoscopic Mayo score from the biopsy site was documented. Biopsies were placed in a standard specimen container which contained sterile gauze, and saline. The specimen containers were immediately transported to the laboratory for processing following their collection.

Each intestinal biopsy was used to generate human UC explants. This biopsy was washed with wash buffer (PBS (Corning, the USA), 1% Pen/Strep (Gibco, Themofisher, the USA), 1% Fungizone (Sigma-Aldrich, the USA), 0.1% Gentamicin (Lonza, Switzerland)) and then cultured in a 24 well plate with media (M199 (Gibco, Themofisher, the USA), 10% FBS (Lonza, Switzerland), 1% Pen/Strep, 1% Fungizone, 0.1% Gentamicin, 1 ug/ml Insulin (Lonza, Switzerland)), and 0.1% Dimethyl sulfoxide (DMSO). Biopsies were incubated for 24 hours at 37 °C with 95% O2/5% CO2 humidified atmosphere to generate human explant-CM. The explant model described in this report has been previously optimised, with assays confirming tissue viability for up to 72 hours.[49,123]

3.3.4. Determination of energy metabolism of explant-CM

In the last hour of culture, colonic biopsies and explant-CM were transferred to an islet capture microplate with capture screens (Agilent Technologies, Santa Clara, California, USA) and incubated in a non-carbon dioxide incubator at 37 °C (Whitley, West Yorkshire, UK) prior to analysis. Seahorse Xfe24 analyser was used to assess metabolic profiles in colonic explants (Agilent Technologies, CA, USA). Following a 12 minute equilibrate step, three basal measurements of OCR (Oxygen Consumption Rate) and ECAR (Extracellular Acidification Rate) were taken over 24 minutes consisting of three repeats of the following sequence "mix (3 minutes)/wait (2 minutes)/measurement (3 minutes)" to establish basal mitochondrial respiration and glycolysis, as well as adenosine triphosphate (ATP) production rate. The OCR measurement represents oxidative phosphorylation and the ECAR measurement

represents glycolysis. Explant-CM was extracted in a sterile environment and tissue was snap frozen. All samples were then stored at −80 °C for further processing.

3.3.5. Determination of secreted inflammatory protein concentrations in explant-CM

Explant-CM secreted proteins were quantified using a V-PLEX Proinflammatory Panel enzyme-linked immunosorbent assay (ELISA) kit (Meso Scale Diagnostics, the USA). These assays quantified the secretions of the following 10 proteins: IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α. All assays were performed according to manufacturer protocol. All secreted protein concentrations were normalised to total protein content of the biopsy tissue specimen by using a BCA assay (Pierce, the USA) as per manufacturer recommendations.

3.3.6. Extraction and quantification of biopsy protein levels

Biopsy tissue specimens were added to cryovials with one metal bead and 200ul RIPA buffer supplemented with EDTA-free protease inhibitor cocktail (Sigma-Aldrich, the USA) and phosphatase inhibitor (Roche, Switzerland). Biopsies were homogenised at 25 Hz for 2 minutes in a tissue lyser (Qiagen, Germany). Samples were transferred to clean Eppendorf tubes and centrifuged for 20 minutes at 1800 rcf at 4 °C. 10ul of each sample was added to 96 well plate in triplicates (Thermo Pierce BCA microassay, the USA) with 200ul of working reagent. The plate was incubated

for 30 minutes at 37 °C in humidified CO2 incubator, and absorbance was read at 562 nm to quantify the total protein content of the sample.

3.3.7.Statistical analysis

Baseline demographic and clinical data were presented as median and interquartile range for continuous variables, and frequencies and percentages for categorical variables. The Mann–Whitney *U* test was used to compare continuous variables between groups by endoscopic remission and advanced therapy status. The Kruskal-Wallis test was used to compare difference in continuous variables between groups for age, metabolism markers and inflammatory proteins. Fisher's exact test was used to compare frequency data. The Kaplan-Meier analysis assessed survival probabilities. Principal Component Analysis (PCA) was performed using the prcomp function in R. Prior to conducting PCA, the data were scaled. Relationships between continuous variables were assessed using Spearman correlation. Correlation heatmaps were generated using the ggplot2 and pheatmap functions in R.

Multivariate linear regression was performed to evaluate the association between ECAR and disease duration. The following covariates were selected due to their biological relevance and included in the model: endoscopic remission status and age at recruitment. Multicollinearity was assessed by generating a variance inflation factor (VIF) for each covariate. A logistic regression model was used to evaluate the independent association between IL-1β levels and the likelihood of experiencing disease progression. Endoscopic remission status was included as a covariate to

control for its potential effect. The model was fitted using the generalized linear model function with a binomial family and a logit link. Odds ratio (OR) and their 95% confidence intervals (CIs) were calculated by exponentiating the model coefficients.

Significance was set at a p-value of 0.05. Statistical analysis was performed using GraphPad Prism 9.5 (GraphPad Software, California, United States) and R Core Team (2023). _R: A Language and Environment for Statistical Computing_. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.

3.4. Results

3.4.1. Baseline characteristics

Twenty-four patients were recruited cross four cohorts: six HC, six IBS patients, six UC patients in remission, and eight UC patients with active disease. Baseline characteristics of the study cohort were similar across all groups. The median age of the overall cohort was 52 years (interquartile range (IQR) 43.5 – 61.25 years). 62% of the overall cohort were male (n=16). There were no differences between cohorts for age and sex (p=0.56 and p=0.27 respectively). The median disease duration for the UC in remission cohort was 16.2 years (3.8 – 20.3), and 9.6 years (5.8 – 15.8) in those with active disease. 33% (n=2) of the UC in remission group were on an advanced therapy at time of endoscopy, whereas 63% (n=5) of the active UC cohort were. There were no differences between the two UC cohorts for disease duration and current biologic used (p= 0.66 and p=0.59 respectively). The majority of both UC cohorts had left-sided disease (67% in UC in remission cohort and 63% in UC with active disease cohort).

Baseline CRP was available for 50% (n=3) of the UC in remission group, and 75% (n=6) of the active UC group. The median CRP at baseline in the remission group was 1 mg/L (IQR 1-4.9), and 2 mg/L (1-5) in the active group (p=0.79). Baseline faecal calprotectin was available for 50% (n=3) of the UC in remission group, and 63% (n=5) of the active UC group. The median faecal calprotectin at baseline in the remission group was 86.6 µg/g (11.5-883) and 48 µg/g (28.6-525.3) in the active disease cohort (p=>0.99). (**Table 12**)

	Control (n=6)	IBS (n=6)	UC (n=14)		P value
			UC in Remission (n=6)	Active UC (n=8)	
Age at scope (median, IQR)	61.5 years (53.7 – 71)	56 years (42.3 – 67.5)	50.5 years (43.5 - 54)	44.5 years (27.1 – 52.8)	0.056
Male sex (%)	5 (83)	2 (33)	2 (33)	4 (50)	0.27
Disease duration (median, IQR)			16.2 years (3.8 – 20.3)	9.6 years (5.8 – 15.8)	0.66
Disease extent (%)			Proctitis 1 (17)	1 (13)	
			Left-sided 4 (67)	5 (63)	
			Pan-colitis 1 (17)	2 (25)	
Current advanced therapy (%)			2 (53)	5 (63)	0.59
Current Anti-TNF therapy (%)			0 (0)	2 (25)	
Endoscopic Mayo score (%)			eMayp 0 4 (67)		
			eMayo 1 2 (33)		
			eMayo 2	6 (75)	
			eMayo 3	2 (25)	
Baseline CRP (median, IQR)			1 mg/L (1-4.9)	2 mg/L (1-5)	0.79
Baseline faecal calprotectin (median, IQR)			86.6 µg/g (11.5-883)	48 µg/g (28.6-525.3)	>0.99

Table 12. Baseline Demographics of healthy controls, UC in remission, and active UC cohorts. No differences can be seen between groups with regards to age and sex. Within UC cohort, no differences are seen with regards to disease duration, distribution, baseline CRP or faecal calprotectin. Anti-TNF, anti-tumour necrosis factor; CRP, C-reactive protein; eMayo, endoscopic Mayo, IBS, irritable bowel syndrome; IQR, interquartile range; UC, ulcerative colitis

3.4.2. Energy Metabolism in UC and IBS

The median oxygen consumption rate (OCR) was significantly higher in the healthy control (HC) cohort (median [IQR]: 571.7 pmol/min [465.2–694.5]) compared to both UC in remission (78.3 pmol/min [43.5–381.9], p = 0.024) and UC with active disease (67.1 pmol/min [17.7–285.5], p = 0.004). In the IBS cohort, OCR levels (211.5 pmol/min [75.9–796.7]) were numerically lower than the HC group but did not reach statistical significance when compared to either HC or UC groups. The median extracellular acidification rate (ECAR) was numerically lower in the HC group compared to IBS, UC in remission, and UC with active disease, although these differences were not statistically significant. The OCR:ECAR ratio was significantly higher in the HC cohort (18.7 [5.9–32]) compared to both UC in remission (2.1 [0.63–6.8], p = 0.013) and UC with active disease (2.7 [0.6–4.4], p = 0.008). There was also a trend towards a lower OCR:ECAR ratio in the IBS group (4.4 [1.4–12.4]) compared to HC, although this did not reach statistical significance (p = 0.09). (**Table 13, Figure 11**).

A sensitivity analysis was undertaken using an alternative definition of active disease, in which Mayo 1–3 (n=10) was considered active and Mayo 0 (n=4) was considered remission. No significant differences in markers of metabolism was observed between the two groups; consistent with the primary analysis.

Energy Metabolism marker (median, [IQR])	Healthy Control	IBS	UC in remission	Active UC
OCR	571.7 pmol/min [465.2 – 694.5]	211.5 pmol/min [75.9 – 796.7]	**78.3 pmol/min [43.5 – 381.9]**	**67.1 pmol/min [17.7 – 285.5]**
ECAR	77.7 pmol/min [44.3 – 177.8]	161.6 pmol/min [96.4 – 246.6]	146.4 pmol/min [99.1 – 325.1]	139.1 pmol/min [74.3 – 322.6]
OCR:ECAR	18.7 [5.9 – 32]	4.4 [1.4 - 12.4]	**2.1 [0.63 – 6.8]**	**2.7 [0.6 - 4.4]**

Table 13. Energy metabolism markers in HC, IBS, UC in remission and UC with active disease. The table shows median levels with interquartile range. Results that are significantly different to HC are marked in bold. Both UC in remission, and UC with active disease had significantly lower OCR and lower OCR:ECAR than HC. There was a non-statistical trend for lower OCR and OCR:ECAR in IBS compared to HC. ECAR, extracellular acidification rate; IBS, irritable bowel syndrome; IQR, interquartile range; OCR, oxygen consumption rate; OCR:ECAR, OCR to ECAR ratio; UC, ulcerative colitis.

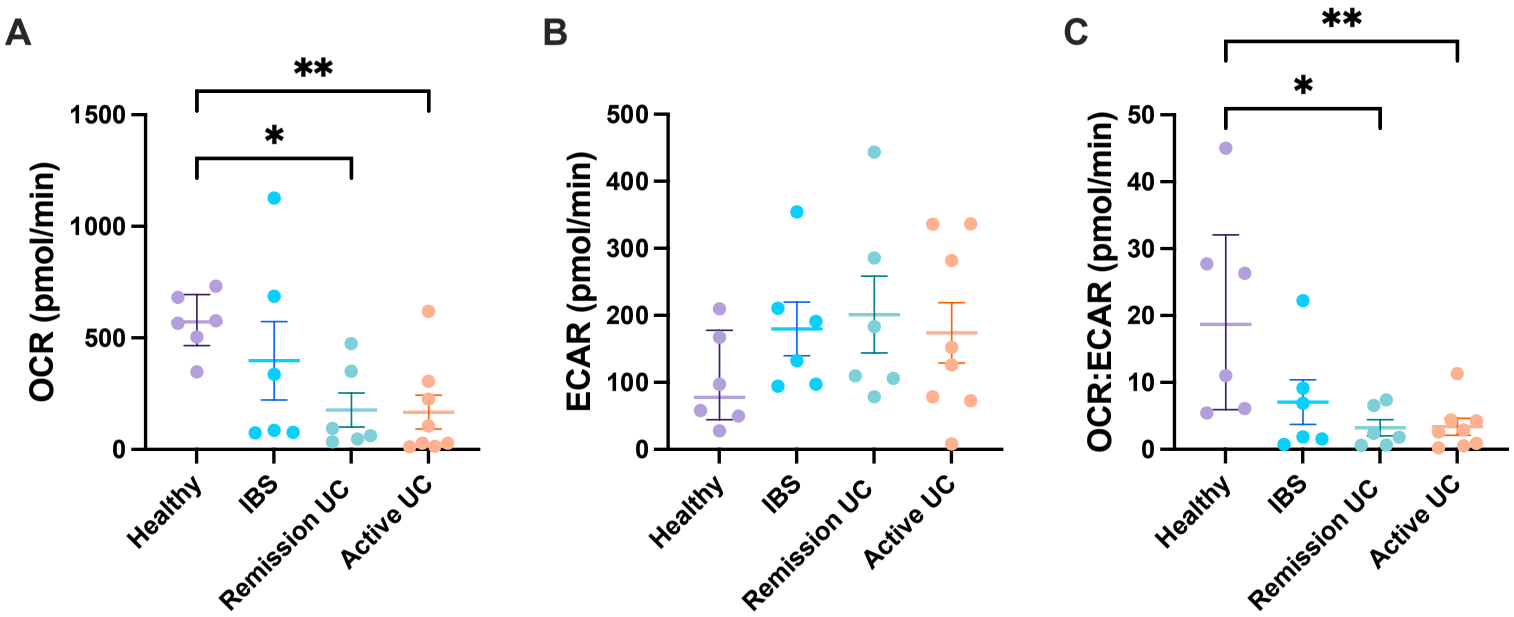


Figure 11. Baseline energy metabolism profiles of four cohorts: Healthy controls, IBS, UC in remission, and active UC.

Graph shows Kruskal-Wallis test, scatter plots (median ± IQR). Statistically significant if P value <0.05, denoted with *, <0.01, denoted with **. ECAR, extracellular acidification rate; IBS, irritable bowel syndrome; OCR, oxygen consumption rate; OCR:ECAR, OCR to ECAR ratio; UC, ulcerative colitis.

Oxygen consumption rate is significantly reduced in both UC in remission and active disease versus healthy controls.

A) Extracellular acidification rate remains unchanged across all cohorts.

B) Oxygen consumption rate:Extracellular acidification rate is significantly reduced in both UC in remission and active disease versus healthy controls.

3.4.3. Inflammatory mediator secretion in UC and IBS

Differences in secreted inflammatory mediators were seen in the active UC cohort compared to other groups. There was a significantly higher median level of IL-4 in the active UC group (0.16 pmol/ml per ug of protein (0.06-0.32)) versus the HC (0.03 pmol/ml per ug of protein (0.02-0.06), p=0.026) and versus IBS (0.03pmol/ml per ug of protein (0.02-0.09), p=0.011) cohorts. Median IL-6 levels were also higher in the UC with active disease cohort (14653 pmol/ml per ug of protein (6165-26826)) compared to HC (222.2 pmol/ml per ug of protein (200-2443), p=0.009) and IBS cohorts (269.6 pmol/ml per ug of protein (101.6-5446), p=0.005). Higher median levels of IFN-γ were seen in the active UC group (4.19 pmol/ml per ug of protein (0.27-44.73)) compared to IBS (0.07 pmol/ml per ug of protein (0.04-0.59), p=0.017) and UC in remission (0.1 pmol/ml per ug of protein (0.02-0.4), p=0.016). Higher median levels of IL-1β were also seen in the active UC group (12.2 pmol/ml per ug of protein (3.4-202.2)) compared to HC (1 pmol/ml per ug of protein (0.8-1.6), p=0.02), IBS (0.45 mol/ml per ug of protein (0.27-1.1), p=0.002) and UC in remission (0.9 mol/ml per ug of protein (0.28-10.4), p=0.031). There were no significant differences in median levels across all cohorts of the remaining inflammatory mediators: IL-2, IL-8, IL-10, IL-12p70, IL-13, and TNF-α. (**Figure 12**).

A sensitivity analysis was undertaken using an alternative definition of active disease, in which Mayo 1–3 (n=10) was considered active and Mayo 0 (n=4) was considered remission. No significant differences in inflammatory protein secretion were observed between the two groups; however, the direction of effect was consistent with the primary analysis.

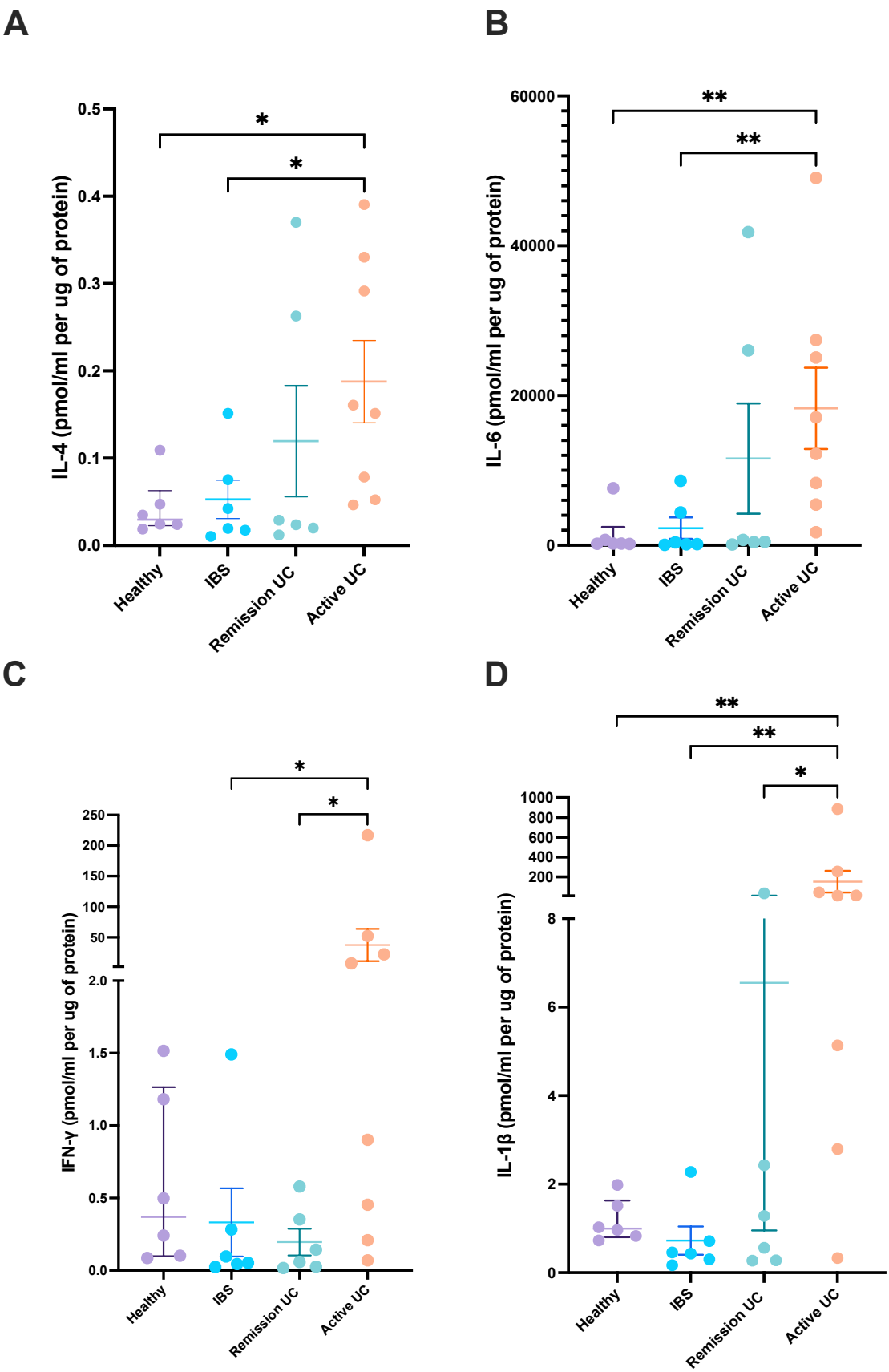

A
IL-4 (pmol/ml per ug of protein)
0.5
0.4
0.3
0.2
0.1
0.0
*
*
Healthy
IBS
Remission UC
Active UC
B
IL-6 (pmol/ml per ug of protein)
60000
40000
20000
0
**
**
Healthy
IBS
Remission UC
Active UC
C
IFN-γ (pmol/ml per ug of protein)
250
200
150
100
50
2.0
1.5
1.0
0.5
0.0
*
*
Healthy
IBS
Remission UC
Active UC
D
IL-1β (pmol/ml per ug of protein)
1000
800
600
400
200
8
6
4
2
0
**
**
*
Healthy
IBS
Remission UC
Active UC

Figure 12. Baseline inflammatory protein secretion with significant differences between the four groups: healthy controls, IBS, UC in remission and active UC. Graph shows Kruskal-Wallis test, scatter plots (median ± IQR). Statistically significant if P value <0.05, denoted with *, <0.01, denoted with **. IBS, irritable bowel syndrome; IL, interleukin; INF, interferon; UC, ulcerative colitis.

A) IL-4 secretion levels. IL-4 levels are significantly higher in active UC versus healthy controls and IBS.

B) IL-6 secretion levels. IL-6 levels are significantly higher in active UC versus healthy controls and IBS.

C) IFN-γ secretion levels. IFN-γ levels are significantly higher in active UC versus IBS and UC in remission.

D) IL-1β secretion levels. IL-1β levels are significantly higher in active UC versus HC, IBS and UC in remission.

3.4.4. Principal Component Analysis of inflammatory-metabolic profiles

The Principal Component Analysis (PCA) biplot illustrates distinct clustering of metabolic and inflammatory profiles across the four cohorts: HC, IBS, UC in remission and active UC. The plot captures 40.8% of variance in the first component (PC1) and 19% in the second component (PC2). While there is some overlapping in the confidence ellipses, there is separation between these groups suggesting differences in inflammatory-metabolic signatures associated with each condition. The HC and IBS clusters exhibit substantial overlap, indicating similar inflammatory-metabolic profiles that distinguish them from the UC groups, with differences seen in metabolic markers associated with the non-UC states, such as OCR. The UC in remission cohort forms a distinct intermediate cluster between HC/IBS and active UC cohorts. The active UC cohort shows the most separation, with characteristic inflammatory and metabolic markers distinguishing it from the other groups: IL-6, IL-8, TNF-α, and IL-13 show stronger associations along PC1, particularly in the regions closer to active UC, suggesting these markers are characteristic of inflammatory activity in active UC. **(Figure 13**)

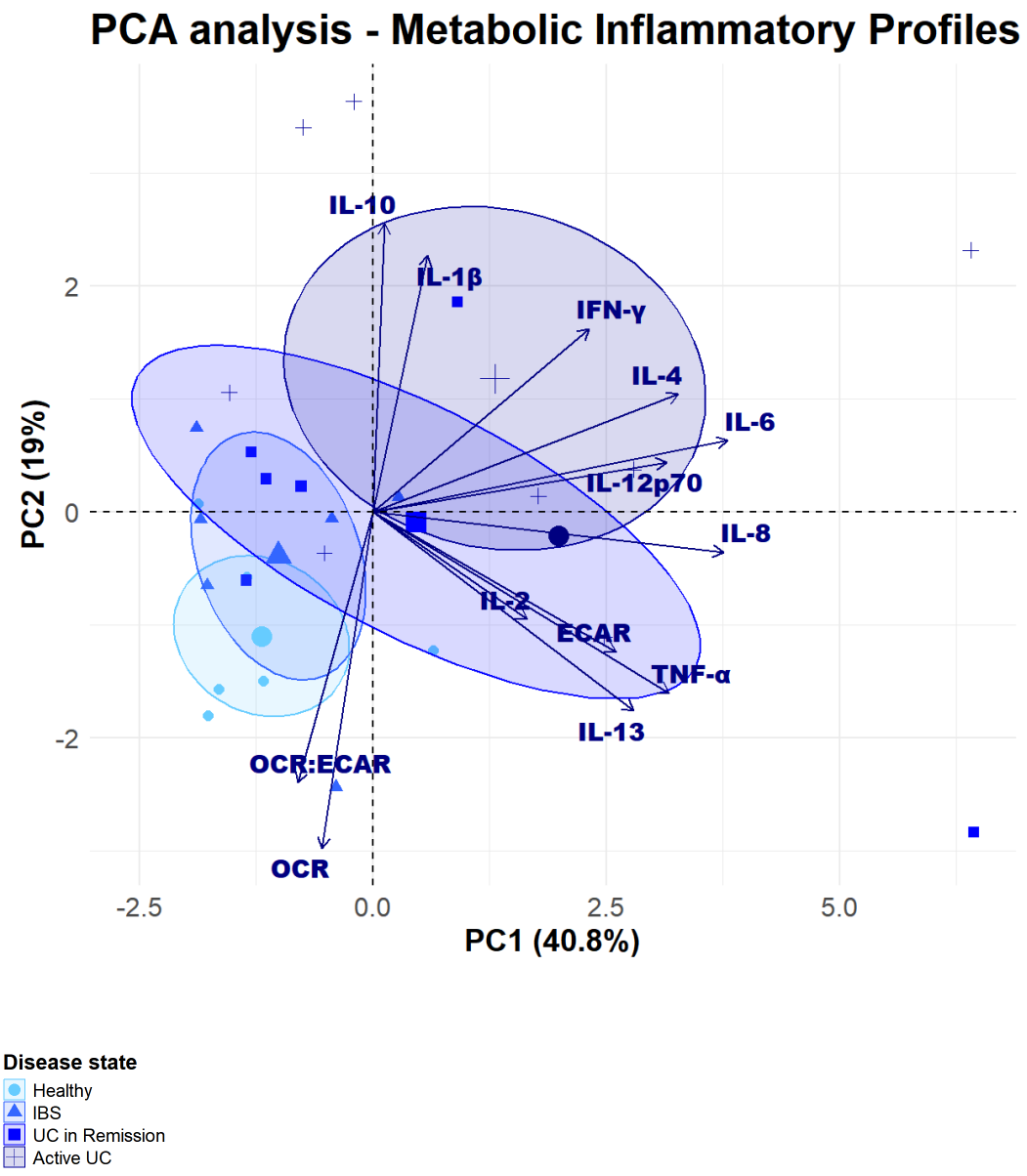
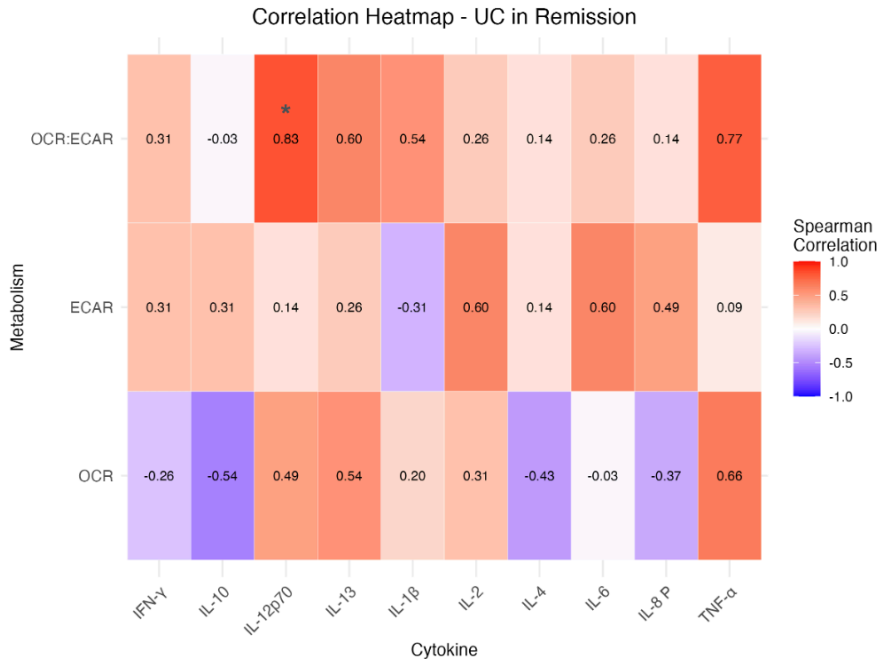


Figure 13. Principle Component Analysis showing active UC has a distinct inflammatory-metabolic profile compared to other groups. HC (light blue circles) are clustered to the left, indicating that their inflammatory-metabolic profiles are distinct from those of other groups. The IBS (blue triangles) occupies a similar region to the healthy group, albeit with some overlap, suggesting partial similarity in profiles with healthy individuals. UC in remission (darker blue squares) is positioned centrally with moderate spread, suggesting intermediate inflammatory-metabolic profiles that share characteristics with HC, IBS and active UC states. The active UC cohort (deep blue squares with grid) spans to the right side of the plot, showing the most separation along PC1 and some overlap with the UC in remission group. This position implies a distinct inflammatory-metabolic profile that is associated with active disease. ECAR, extracellular acidification rate; HC, healthy control; IBS, irritable bowel syndrome; IL, interleukin; INF, interferon; OCR, oxygen consumption rate; OCR:ECAR, OCR to ECAR ratio; PC, principal component; TNF, tumour necrosis factor; UC, ulcerative colitis.

3.4.5. Association between energy metabolism markers and inflammatory mediator secretion

The OCR:ECAR is positively correlated with IL-12p70 in the UC in remission cohort ($\rho = 0.83$, $p = 0.04$). ECAR is positively correlated with IL-12p70 and IL-8P in the active UC cohort ($\rho = 0.83$, $p = 0.01$, and $\rho = 0.79$, $p = 0.02$ respectively). (**Figure 14**) In the non-UC cohorts, OCR is positively correlated with IL-1β and IL-2 ($\rho = 0.83$, $p = 0.04$, and $\rho = 0.83$, $p = 0.04$ respectively) in the HC cohort, and ECAR is positively correlated with TNF-α ($\rho = 0.83$, $p = 0.04$) in the IBS cohort. (**Figure 15**)

A


Correlation Heatmap - UC in Remission
OCR:ECAR
ECAR
OCR
Metabolism
0.31 -0.03 * 0.83 0.60 0.54 0.26 0.14 0.26 0.14 0.77
0.31 0.31 0.14 0.26 -0.31 0.60 0.14 0.60 0.49 0.09
-0.26 -0.54 0.49 0.54 0.20 0.31 -0.43 -0.03 -0.37 0.66
IFN-γ
IL-10
IL-12p70
IL-13
IL-1β
IL-2
IL-4
IL-6
IL-8 P
TNF-α
Cytokine
Spearman Correlation
1.0
0.5
0.0
-0.5
-1.0

B

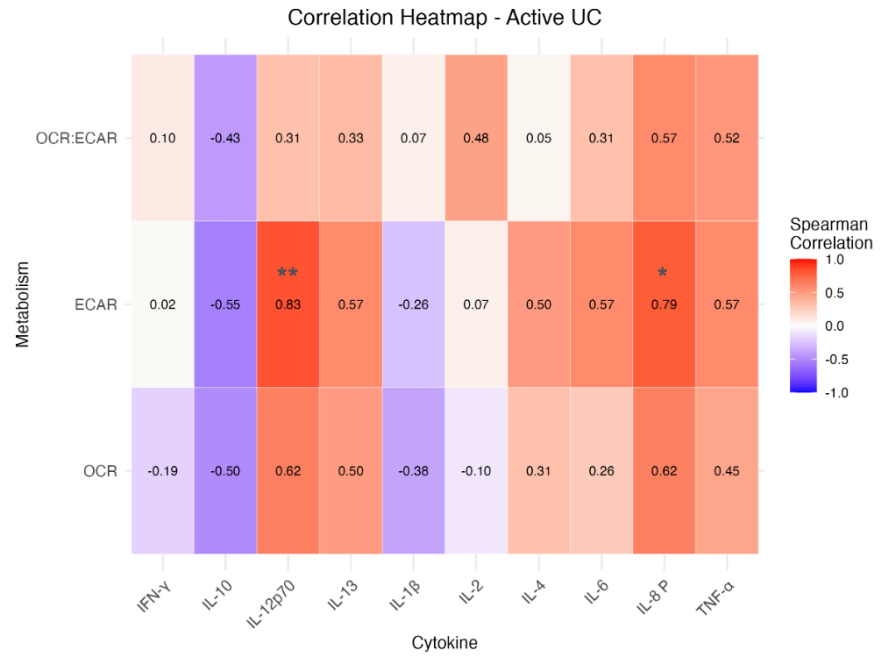

Correlation Heatmap - Active UC
OCR:ECAR
ECAR
OCR
Metabolism
0.10 -0.43 0.31 0.33 0.07 0.48 0.05 0.31 0.57 0.52
0.02 -0.55 ** 0.83 0.57 -0.26 0.07 0.50 0.57 * 0.79 0.57
-0.19 -0.50 0.62 0.50 -0.38 -0.10 0.31 0.26 0.62 0.45
IFN-γ
IL-10
IL-12p70
IL-13
IL-1β
IL-2
IL-4
IL-6
IL-8 P
TNF-α
Cytokine
Spearman Correlation
1.0
0.5
0.0
-0.5
-1.0

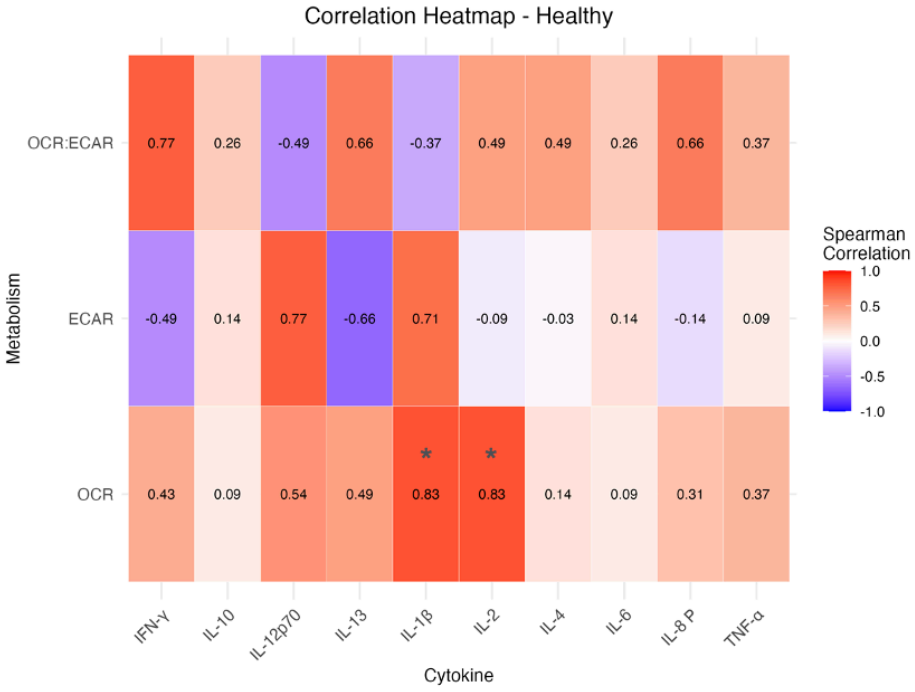
Figure 14. Heatmaps of correlation between energy metabolism markers (OCR, ECAR, OCR:ECAR) and cytokine secretion for A) UC in remission, and B) active UC.

Each matrix displays the correlation coefficients, with colour intensity representing the strength and direction of the correlations (red for positive and blue for negative correlations). Statistically significant correlations marked with "*". ECAR, extracellular acidification rate; IL, interleukin; INF, interferon; OCR, oxygen consumption rate; OCR:ECAR, OCR to ECAR ratio; TNF, tumour necrosis factor; UC, ulcerative colitis.

A) OCR:ECAR is positively correlated with IL-12p70 ($\rho = 0.83$, $p = 0.04$) in UC in remission.

B) ECAR is positively correlated with IL-12p70 and IL-8P ($\rho = 0.83$, $p = 0.01$, and $\rho = 0.79$, $p = 0.02$ respectively) in active UC.

A


Correlation Heatmap - Healthy
OCR:ECAR
ECAR
OCR
Metabolism
0.77 0.26 -0.49 0.66 -0.37 0.49 0.49 0.26 0.66 0.37
-0.49 0.14 0.77 -0.66 0.71 -0.09 -0.03 0.14 -0.14 0.09
0.43 0.09 0.54 0.49 * 0.83 * 0.83 0.14 0.09 0.31 0.37
IFN-γ
IL-10
IL-12p70
IL-13
IL-1β
IL-2
IL-4
IL-6
IL-8 P
TNF-α
Cytokine
Spearman Correlation
1.0
0.5
0.0
-0.5
-1.0

B

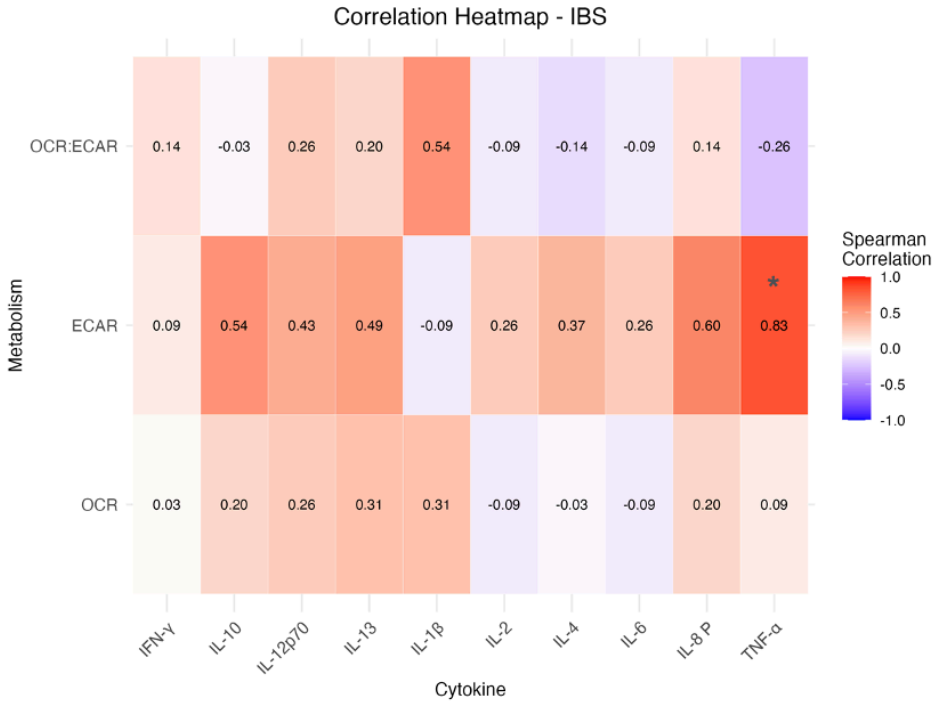

Correlation Heatmap - IBS
OCR:ECAR
ECAR
OCR
Metabolism
0.14 -0.03 0.26 0.20 0.54 -0.09 -0.14 -0.09 0.14 -0.26
0.09 0.54 0.43 0.49 -0.09 0.26 0.37 0.26 0.60 * 0.83
0.03 0.20 0.26 0.31 0.31 -0.09 -0.03 -0.09 0.20 0.09
IFN-γ
IL-10
IL-12p70
IL-13
IL-1β
IL-2
IL-4
IL-6
IL-8 P
TNF-α
Cytokine
Spearman Correlation
1.0
0.5
0.0
-0.5
-1.0

Figure 15. Heatmaps of energy metabolism markers (OCR, ECAR, OCR:ECAR) and cytokine secretion for A) Healthy controls, and B) IBS.

Each matrix displays the correlation coefficients, with colour intensity representing the strength and direction of the correlations (red for positive and blue for negative correlations). Statistically significant correlations marked with "*". ECAR, extracellular acidification rate; HC, healthy control; IBS, irritable bowel syndrome; IL, interleukin; INF, interferon; OCR, oxygen consumption rate; OCR:ECAR, OCR to ECAR ratio; TNF, tumour necrosis factor.

A) OCR is positively correlated with IL-1β and IL-2 ($\rho = 0.83$, $p = 0.04$, and $\rho = 0.83$, $p = 0.04$ respectively) in healthy controls.
B) ECAR is positively correlated with TNF-α ($\rho = 0.83$, $p = 0.04$) in IBS.

3.4.6. Association between baseline characteristics and inflammatory and energy metabolism markers

There was no correlation between age at endoscopy and energy metabolism markers in the overall cohort. (**Figure 16**) In the overall UC cohort there was no difference in energy metabolism markers and remission status or current advanced therapy use. (**Figure 17 & 18**) However, ECAR positively correlated with disease duration (ρ=062, p = 0.02). (**Figure 19**) A multivariate regression analysis, which included ECAR, endoscopic remission status, and age at recruitment, demonstrated that ECAR was independently associated with UC disease duration, (standardised beta 0.03, p=0.043). No inflammatory markers correlated with disease duration. In the overall UC group IL-10 positively correlated with baseline faecal calprotectin (ρ=0.88, p=0.003), while IL-2 negatively correlated with baseline CRP (ρ = -0.57, p=0.035). (**Figure 20**) Metabolism markers did not correlate with these biochemical markers.

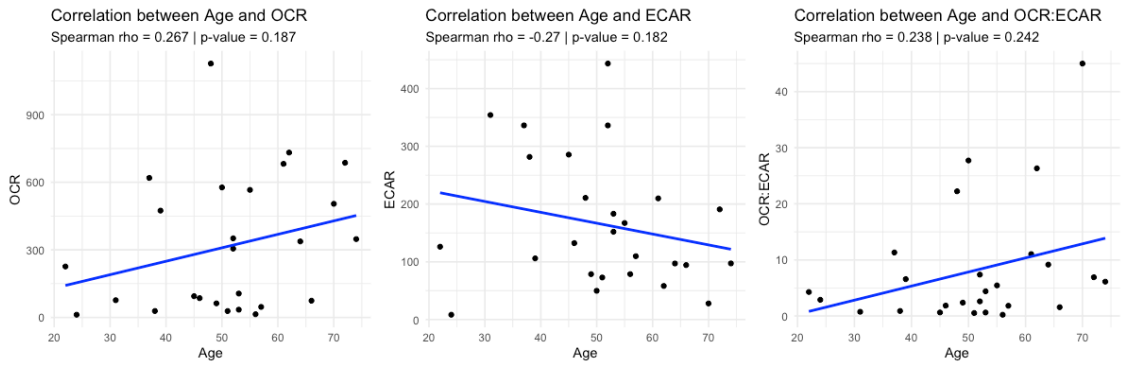


Figure 16. No correlation is observed between age at endoscopy and energy metabolism markers. Spearman correlation showing no correlation between age at endoscopy and energy metabolism markers. ECAR, extracellular acidification rate; OCR, oxygen consumption rate; OCR:ECAR, OCR to ECAR ratio.

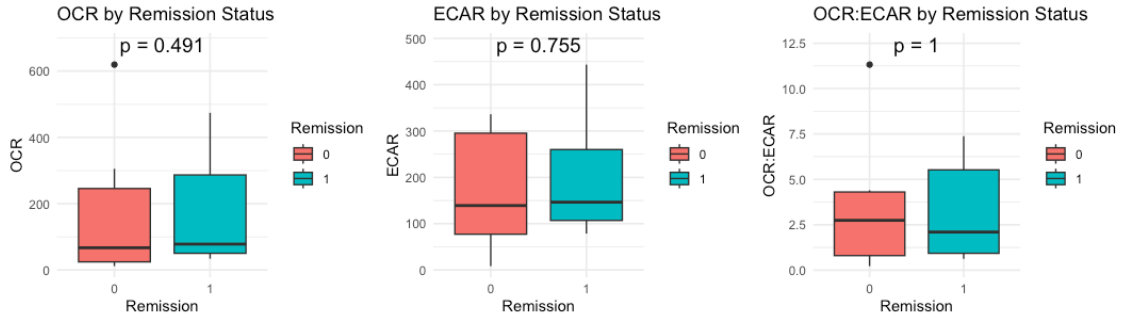


Figure 17: Comparison of energy metabolism markers between remission and active disease groups. The Mann-Whitney U-Test revealed no significant difference in the mean ranks of energy metabolism markers between patients in remission and those with active disease. "0" signifies no remission, "1" signifies remission. ECAR, extracellular acidification rate; OCR, oxygen consumption rate; OCR:ECAR, OCR to ECAR ratio.

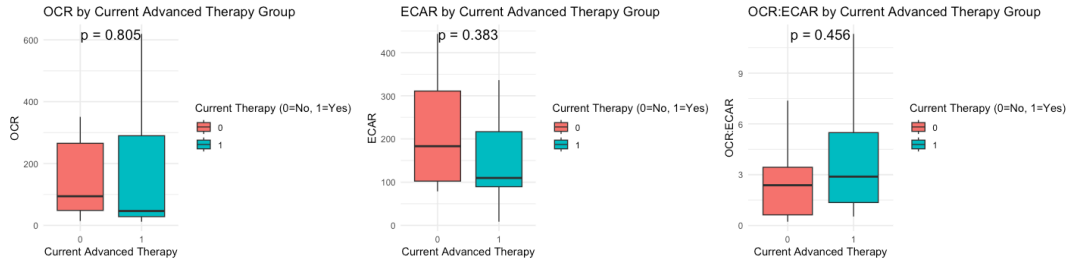


Figure 18. Comparison of energy metabolism markers between UC patients on an advanced therapy and those not on an advanced therapy currently. The Mann-Whitney U-Test revealed no significant difference in the mean ranks of energy metabolism markers between patients currently on an advanced therapy and those not on an advanced therapy. "0" signifies no advanced therapy, "1" signifies advanced therapy. ECAR, extracellular acidification rate; OCR, oxygen consumption rate; OCR:ECAR, OCR to ECAR ratio; UC, ulcerative colitis.

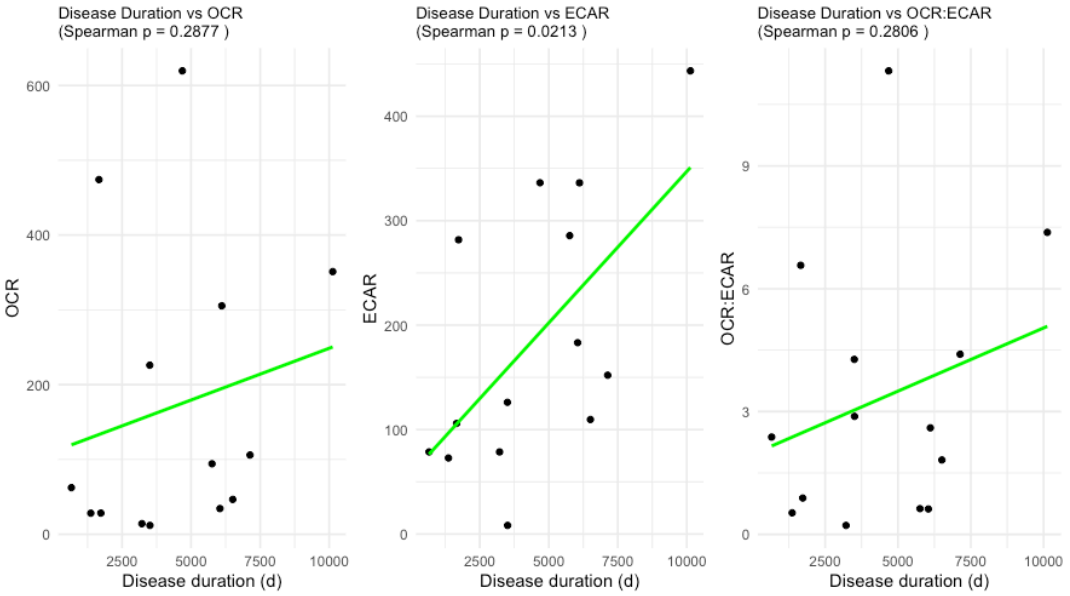


Figure 19. Correlation between metabolism markers and disease duration. ECAR is positively correlated with disease duration (p = 0.02). Multivariate analysis, including endoscopic remission status, and age at recruitment, demonstrated increased ECAR to be independently associated with UC disease duration, p = 0.043. ECAR, extracellular acidification rate; OCR, oxygen consumption rate; OCR:ECAR, OCR to ECAR ratio; UC, ulcerative colitis.

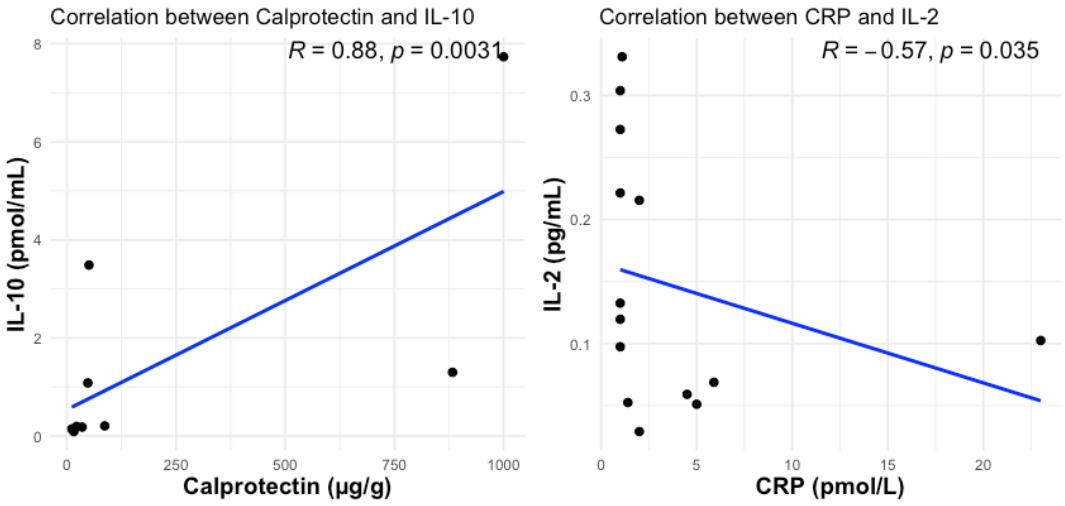


Figure 20. Correlation between UC biomarkers and explant cytokine secretion. In the UC group IL-10 positively correlated with baseline faecal calprotectin (ρ=0.88, p=0.003), while IL-2 negatively correlated with baseline CRP (ρ = -0.57, p=0.035). IL, interleukin; CRP, C-reactive protein.

3.4.7. UC disease progression

During the follow-up period 35.7% (n=5) of the overall cohort had disease progression. The median time to disease progression or last follow-up was 26.2 weeks (IQR, 3-50). 16.7% (n=1) of the remission group progressed, whereas 50% (n=4) of patients from the active disease group had disease progression. However, there was no significant difference in numbers who progressed from each group nor in time to progression (p=0.3 and p=0.13).

There was no association between the metabolism markers and disease progression. Higher levels of IL-1β were seen in patients with disease progression than in those without (median, (IQR), 44.6 pmol/ml per ug of protein (6.9 – 570) versus 1.3 pmol/ml per ug of protein (0.3 – 9), p=0.29). Logistic regression analysis, which included endoscopic remission status as a covariate, demonstrated a positive but non-significant association between IL-1β levels and disease progression status (OR = 1.06, 95% CI: 1.00–1.19, p = 0.244). Remission status (OR = 0.51, 95% CI: 0.02–10.32, p = 0.654) was not significantly associated with flare. (**Figure 21**)

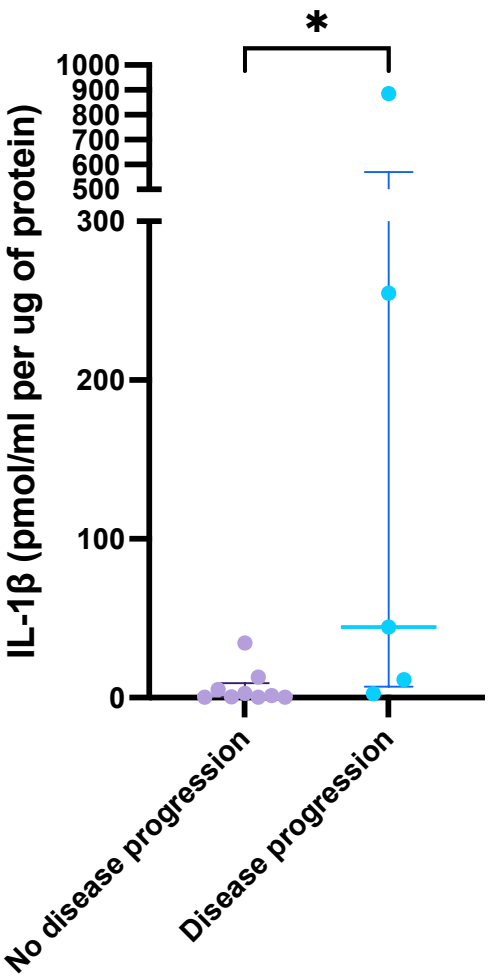


Figure 21. IL-1β secretion was increased in UC patients with disease progression compared to those without. Graph shows Mann-Whitney U test, scatter plot (median ± IQR). Statistically significant P value of <0.05, denoted with *. Logistic regression analysis demonstrated a positive but non-significant association between IL-1β levels and progression status (OR = 1.06, 95% CI: 1.00–1.19, p = 0.244). IL, interleukin; IQR, interquartile ration; OR, odd ratio; UC, ulcerative colitis.

3.5. Discussion

In this report, we have used the human ex vivo colonic explant model to evaluate differences in inflammatory and metabolic profiles of HC tissue compared to IBS or UC tissue. The findings reveal distinct inflammatory and metabolic changes in UC, particularly in active disease, while IBS demonstrated only subtle differences compared to HC.

We demonstrate that active UC is associated with increased production of multiple pro-inflammatory cytokines, including IL-4, IL-6, IFN-γ and IL-1β. Assessment of UC mucosa bioenergetics demonstrated a reduction in oxidative phosphorylation in the UC cohort compared to HC, regardless of remission status, as determined by a low OCR. Further to this, there was an associated shift in cellular metabolism towards glycolysis, as determined by a low OCR:ECAR in the UC cohort. In inactive UC, the OCR:ECAR positively correlated with IL-12p70 levels. In active disease, ECAR positively correlated with IL-8 and IL-12p70 secretion, suggesting an increasing reliance on glycolysis as inflammation increases. IL-12p70 is not itself a direct glycolytic target, however inhibition of glycolysis has been shown to suppress IL-12p70 secretion, supporting the interpretation that our observed correlation with ECAR reflects a metabolically regulated inflammatory axis rather than an incidental association.[124] Notably, ECAR was found to increase as disease duration lengthens, independent of current inflammation, suggesting an increasing use of glycolysis for energy production as the disease course lengthens. Although there was no significant correlation between disease duration and cytokine production, there was a non-statistical trend between increasing IL-2, IL-4 and IL-8p production and longer disease duration. Faecal calprotectin levels positively associated with IL-10 levels, whereas CRP negatively associated with IL-2. IL-1β

levels were higher in those that had disease progression; however, multivariate logistical regression showed that IL-1β was not independently associated with disease progression.

We did not demonstrate a difference in inflammatory protein secretion in IBS tissue compared to HC, nor differences in metabolism biomarkers. However, PCA analysis shows some separation between the IBS and HC groups, suggesting some differences in the inflammatory and metabolic profiles. Notably, there was a non-significant trend for lower OCR and OCR:ECAR in IBS compared to HC. This may suggest reduced oxidative phosphorylation and an increased reliance on glycolysis for energy production in IBS.

Elevated mucosal levels of IL-6 and IL-1β are well described in active UC. A 1996 study by Reimund et al, using organ cultures of UC and CD mucosa, demonstrated IL-1β and IL-6, in addition to TNF-α, to be significantly elevated in inflamed areas of IBD mucosa compared to non-inflamed areas.[125-127] IL-6 and IL-1β are now understood to be key initiators of colitis, and drugs inhibiting both pathways have been developed and have undergone early clinical trial testing in UC.

Inhibition of IL-6, by drugs such as Tocilizumab, causes significant immunosuppression. As such Olamkicept, a selective IL-6 trans-signalling inhibitor with a reduced risk of immunosuppression, has been developed for treatment of UC. Results from a phase 2 study regarding the use of in UC have recently been published with higher doses showing an increased likelihood of clinical response at 12 weeks versus placebo.[128,129] Results from a phase 2 study regarding the use of Anakinra, an IL-1α and IL-1β inhibitor already licensed for

rheumatoid arthritis, for acute severe ulcerative colitis have recently been presented. Anikara was added current standard therapy alongside steroids. The trial was terminated on futility grounds as no reduction in the need for rescue therapy or colectomy in the Anikara treatment arm was observed.[51,130,131]

IFN-γ appears to play a role in the pathogenesis of UC, with murine models suggesting it disrupts vascular barriers and contributes to intestinal inflammation. There is conflicting evidence about its secretion in inactive and active disease states. Pearl et al. demonstrated in a 2013 study that IFN-γ levels were significantly reduced in inflamed mucosa compared to non-inflamed mucosa in UC patients. More recently, Butera et al used quantitative PCR to analyse the mucosal cytokine messenger RNA (mRNA) content of both inflamed and non-inflamed UC tissue from the same patient, and from control samples. They found that IFN-γ levels were significantly elevated in both inflamed and non-inflamed UC tissue compared to control samples, with the highest levels of IFN-γ observed in inflamed tissue. IFN-γ mRNA levels also showed strong correlations with other pro-inflammatory cytokines, including IL-6. These more recent findings are in keeping with our results that indicate that IFN-γ plays a role in active UC. Interestingly, inhibition of IFN-γ signalling in endothelial cells ameliorated colitis in a mouse model, suggesting it may be a therapeutic target.[52,132-137]

Active UC is a T helper (Th) 1 cell-mediated disease in the early phases, changing to a Th2-driven disease in later phases. Animal studies have shown that cytokine production cytokine profiles vary across these disease stages. At disease onset, cytokines derived from innate immune cells, such as TNF-α, IL-1β, and IL-6, predominate. This is followed by a predominance of Th1-associated cytokines in the early phase and Th2-associated cytokines in the later phase. Th17-related

cytokines appear to contribute during both phases of disease.[133] These findings have been replicated in human studies where Th1- and Th2-related genes were associated with early disease and late disease (>10 years), respectively.[138] IL-4 is a Th-2-type cytokine that promotes wound healing.[75,138] IL-4 mRNA expression has been shown to be higher in UC patients than controls, with expression more frequent in active disease. It has previously been shown to increase progressively from pre- to early to late chronic phases of disease in IL-10 deficient and T cell receptor alpha chain-deficient (Tcra -/-) mice.[139,140] Further to this, it has been shown to be upregulated in mucosal samples from active UC patients with late disease. We demonstrated that IL-4 was increased in the active UC cohort with a non-statistical trend between increasing IL-4 production and longer disease duration. These findings highlight IL-4 as a potential therapeutic target in treating UC. However, results from a mouse model of UC, with IL-4Rα deletions and subsequent loss of IL-4Rα signalling on intestinal epithelial cells, smooth muscle cells and macrophages/neutrophils, had no effect on alleviating acute colitis. The effect of loss of IL-4Rα signalling on chronic colitis was not tested. On the other hand, a proof-of-concept study demonstrated that human IL-4–treated regulatory macrophages promoted epithelial wound healing and reduced chronic colitis in murine models through transforming growth factor-β (TGF-β) mediated mechanisms. These findings underscore the complex role of IL-4 in UC pathogenesis and highlight the importance of personalised therapeutic strategies that consider the disease phase.[52,132,133,141]

Calprotectin, a reliable clinical biomarker for IBD and colitis, is a cytosolic protein complex that is expressed in neutrophils. It can induce secretion of pro-inflammatory cytokines such at IL-1β, IL-6 and TNF-α in association with oxidative

stress. Yang et al have demonstrated that overexpression of the calprotectin complex by macrophages stimulates homeostatic IL-10 secretion as part of an anti-inflammatory feedback mechanism. Our findings support this work, showing association between calprotectin and IL-10 in an ex vivo model, reflecting the functional role this biomarker plays in vivo.[35,142]

IL-2 is involved with intestinal immune regulation primarily through its role in shaping the regulatory T cell pool, with IL-2 deficiency leading to spontaneous colitis in mice. Mutations in the IL-2 pathway are linked to colitis and Mendelian forms of IBD. While there are no previously published correlations between systemic CRP and mucosal IL-2 secretion, our data, which show a negative correlation between explant IL-2 secretion and CRP, are biologically consistent. This finding highlights IL-2 as a potential novel therapeutic pathway in treating UC. Indeed, recent work published by the Low-Dose IL2 UC Study Group has shown promising results of a phase 1b/2a study using low-dose IL-2 as a treatment for moderate to severe UC, where clinical remission was seen in up to 30.8% of patients at week 8.[75,143-145]

Mitochondrial function in the intestinal epithelium plays a critical role in maintaining intestinal health, ensuring energy production, supporting epithelial barrier integrity and regulating immune responses. Dysfunction in mitochondrial metabolism is increasingly recognised as a key contributor to intestinal inflammation and the pathogenesis of diseases such as UC.[146] Schneider et al recently published work supporting our data demonstrating reduced oxidative phosphorylation (OXPHOS) in UC and an increased reliance on glycolysis in certain cohorts. Through staining colonic biopsies immunohistochemically for five

OXPHOS complexes, a reduced expression of mitochondrial OXPHOS complexes was observed in the rectal mucosa of UC and CD patients compared to controls. Adult UC patients in the study showed a more pronounced reduction in mitochondrial mass and OXPHOS complex expression compared to paediatric or CD patients. The analysis did not account for UC disease duration, which was found to be independently associated with increasing glycolysis in our study.[73,120,73,147]

Targeting metabolism has come under increased scrutiny in treating IBD and other diseases. Reducing glycolysis and enhancing mitochondrial respiration has been shown to reverse the Warburg effect in cancer cells. This is now an emerging treatment for various solid tumours, where clinical trials are ongoing.[148] Bär et al showed in murine models that alterations in mitochondrial DNA that augment oxidative phosphorylation and increase mucosal ATP protect the mice from colitis. Colonic tissue from these mice showed increased enterocyte proliferation and transcription factor nuclear factor-kB activity. Aberrations in both factors have been linked with impaired gut barrier function and implicated with IBD.[149] In a dextran sulfate sodium (DSS)-induced colitis mouse model, sodium-glucose co-transporter 2 (SGLT2) inhibitors reduced inflammation by inhibiting glycolysis and cytokine production via their suppressive action on M1 macrophage polarisation. Further to this glycolysis is known to influence the gut microbiome, diminishing SCFA-producing beneficial bacteria and thus impairing gut barrier function.[150] Our study has demonstrated significantly dysregulated metabolism in both active and inactive UC, suggesting treatments that target mitochondrial bioenergetics might play a role not only in treating colitis, but also in altering the underlying pathophysiology of disease when in remission. Additionally, we demonstrated an

increasing rate of glycolysis associated with pro-inflammatory cytokines (IL12p70 and IL-8), suggesting an association between increasing glycolysis and increasing inflammation, further suggesting inhibition of glycolysis as a novel treatment target in UC.[65151,152]

We did not find a significant association between energy metabolism biomarkers and the risk of disease relapse in UC. However, previous studies have suggested that mitochondrial metabolism plays a role in influencing disease outcomes. In particular, increased expression of antiapoptotic factors belonging to the MTRNR2-like gene family and non-coding RNAs have been associated with longer remission durations and reduced relapse risk. While our data did not demonstrate this relationship, larger studies with longer follow-up periods are required to further investigate the role of mitochondrial function and bioenergetics in predicting disease progression and relapse in UC, and whether the observed dysfunction can be reversed or resolves over time.[153]

Previous research has shown a reduction in IL-10 levels in the mucosa of patients with IBS diarrhoea predominant or postinfectious IBS with some consistency. There is a lack of consensus as to whether numbers of T cells and other cytokine levels are abnormal in this disease state.[52] We did not demonstrate a difference in inflammatory protein secretion in IBS tissue compared to HC. The observed differences in inflammatory protein secretion between UC and IBS reflects the well-established divergence in their underlying immunopathology: UC is characterised by expansion and activation of pro-inflammatory immune cells, whereas IBS typically exhibits preserved cellular composition with only low-grade, T cell and mast cell immune activation compared to HC.[133,154]

While cellular metabolism has been extensively studied in UC, there is currently limited research published on this topic in the field of IBS. There was a lower numerical rate of OXPHOS in the IBS patients compared to the HC, suggesting a potential role for mitochondrial dysfunction in IBS pathophysiology. This is supported by recent work demonstrating higher mitochondrial DNA copy numbers in IBS patients, correlating with symptom severity. A further study identified mitochondrial DNA polymorphisms, previously linked to other functional disorders, to be present in a subgroup of IBS patients. Further research into mitochondrial metabolism in IBS is warranted.[78,155]

The clinical impact of our findings includes identification of a number of treatment targets in UC. These include targets already under assessment such inhibition of IL-6, and IL-1β, and more novel targets such as inhibition of IFN-γ, IL-4, IL-2 and glycolysis or targeted stimulation of OXPHOS. Our study builds on the evolving body of evidence regarding the role of mitochondrial dysfunction in IBD pathogenesis. The progressive shift in methods of energy production associated with disease duration in UC is previously unreported and suggests different targeted therapies may be beneficial at different stages in the disease course. Increasing mitochondrial dysfunction may contribute to the progressive histological and functional features associated with UC. Understanding and targeting these features are key to preventing negatively impacting disease factors associated with longstanding UC. Future studies with larger patient cohorts and longer follow-up will be essential to confirm these findings and elucidate the role of mitochondrial bioenergetics in UC disease progression and relapse. Our findings support the use of the explant model as a powerful tool for precision medicine. The

explant model enables the assessment of an individual's cytokine responses at a certain disease stage, and perhaps more targeted inhibition of these cytokines.

The absence of significant cytokine abnormalities associated with IBS observed in our study may be attributed to the small sample size and heterogeneity of the cohort. However, this finding does not definitively rule out the possibility of a limited inflammatory basis underlying certain cases of IBS. It also highlights the potential role mitochondrial metabolism may play in IBS pathophysiology.

The primary limitation of this study was sample size, which necessitated defining endoscopic remission as Mayo 0–1; while exploratory analyses was completed using a stricter Mayo 0 definition, the very small numbers precluded robust statistical comparison. Additionally the observed differences in cytokine expression between the active UC and UC in remission groups give support to this subclassification of disease activity. Despite this, we were able to show distinct differences between the UC cohort and the HC, which differences were biologically consistent with the known disease pathophysiology. Principal Component Analysis showed the IBS profile to be somewhat different to the HC; however, given the small sample size it was not possible to further elucidate this with regard to metabolism and cytokine release. A further limitation of this study was the significant heterogeneity of patient disease type and treatment exposure history. The UC patients had varying rates of advanced therapy exposure. It is not fully understood how exposure to these therapies affects the UC microenvironment. Additionally, there was no paired histological analysis of the study biopsies. As a result, we relied on endoscopic classification for the UC cohort. There was a lack of

sub-classification of the IBS cohort by diarrhoea and constipation phenotype, which may have impacted the lack of inflammatory protein secretion differences seen between this group and HC.

3.6. Conclusion

In conclusion, we have further validated the human ex vivo explant model in IBD. This model has been shown to accurately capture the IBD microenvironment. It has yielded novel insights into the crosstalk between metabolism and inflammatory signallers, thus reflecting the complexity of the IBD microenvironment. Through this model, we have shown that HC, IBS, UC in remission and active UC have differences in their inflammatory and metabolic profiles. Mitochondrial dysfunction seen in UC increases over time, independent of inflammatory activity, suggesting that this dysfunction plays a role in UC pathogenesis. Our findings support further exploration of targeted therapies towards cellular metabolism in UC.

3.7. Summary of findings of chapter 3

In this chapter, we described different inflammatory and metabolic profiles associated with HC, UC patients and IBS patients using a colonic explant model. Active UC was demonstrated to have increased production of the pro-inflammatory mediators, IL-4, IL-6, IFN-γ and IL-1β. We report a previously unreported finding of correlation between faecal calprotectin levels and explant IL-10 secretion, likely reflecting a homeostatic role of calprotectin. Assessment of UC mucosa bioenergetics demonstrated a reduction in oxidative phosphorylation regardless of remission status. Increasing rates of glycolysis were associated with

pro-inflammatory cytokine secretion. Rates of glycolysis were found to increase as disease duration lengthens, independent of current inflammation and age. These findings represent novel therapeutic targets for drug discovery and add to our understanding of the pathophysiology of IBD.

Differences in inflammatory protein secretion were not demonstrated in IBS tissue compared to HC tissue. However, we noted a trend of reduced oxidative phosphorylation and increased reliance on glycolysis for energy production in IBS. This may represent a new treatment target; however, further research is required to elucidate the exact role mitochondrial bioenergetics plays in IBS.

Chapter 4: The Effects of Infliximab and Natural Plant Extracts on the Inflammatory and Metabolic Profiles of Ulcerative Colitis & Irritable Bowel Syndrome

4.1. Aims of chapter 4

The overall aim of this chapter is to determine whether established or novel candidate therapeutic plant extracts alter the inflammatory and metabolic profiles in HC, UC patients and IBS patients.

Specific aims include:

1. To determine whether treatment of UC explants with infliximab alters inflammatory protein secretion (IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α) or baseline metabolism markers (OCR, ECAR, OCAR:ECAR).

2. To determine whether treatment of HC, UC or IBS explants with cuckoo flower, tormentil or cross-leaved heath alters inflammatory protein secretion (IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α) or baseline metabolism markers (OCR, ECAR, OCAR:ECAR).

3. To determine whether overall inflammatory and metabolic explant profiles are altered by candidate drugs using Principal Component Analysis.

4.2. Introduction

Inflammatory bowel disease (IBD), comprised of ulcerative colitis (UC) and Crohn's Disease, and irritable bowel syndrome (IBS), are common digestive disorders. As noted above, while originally thought to represent opposite ends of the spectrum as regards inflammatory versus non-inflammatory mediated gastrointestinal diseases, there is now an evolving body of evidence of significant overlap in the pathophysiology of both diseases. Both have been associated with impaired immune regulation and barrier function, genetic predisposition, and alterations in microbial form and function.[4,52]

There have been considerable breakthroughs of new treatment options for IBD in recent years. However despite this there remains a ceiling of treatment efficacy whereby induction remission rates remain as low as 20–30% in the pivotal clinical trials for UC, with a considerable proportion losing response over time.[23] There are limited treatment options for mild IBD. 5-aminosalicylic acid remains the primary treatment of mild to moderate UC; however, this is of limited benefit in CD.[22,156] Evidently, new therapeutic targets are required. There has been increasing interest in targeting cellular metabolism as one such novel target.[157]

IBS represents a heterogenous constellation of symptoms. Treatments vary from pharmacological, microbial manipulation, complementary therapies and exercise to dietary interventions. Many of the clinical trials in this area have been untargeted and lack mechanistic evidence for the drug in question. There is an unmet need for further pharmacological interventions with mechanistic backing for this disease.[56]

Natural products have been key to drug discovery and advances in treatment over the past century, discovering treatments for conditions ranging from infection to cancer.[87] Up to 43% of patients with digestive diseases use complementary therapies, many of which are natural products.[158] The National Center for Complementary and Integrative Health defines complementary health approaches as a "group of diverse medical and health care systems, practices, and products whose origins come from outside of mainstream medicine", which includes herbal remedies and other dietary supplements.[8] These approaches are commonly used by patients with IBD and IBS, with or without conventional therapy. Patients find complementary approaches highly attractive, citing fewer concerns regarding safety and side effects compared to conventional approaches. While patients might perceive these natural therapies to be less toxic, there is often a lack of knowledge regarding the mechanisms of actions of these therapies, potential adverse reactions and effectiveness. As such there is a need for high quality scientific research in this area. [88,89,158,159]

'Unlocking Nature's Pharmacy from Bogland Species', led by the Trinity Centre of Natural Products Research, is a project investigating the potential therapeutic and commercial use of native Irish bog plants and distinctive bog waters.[8] Peat bogs, moors and heathlands cover nearly 16% of Irish land. The European Union has designated them to be nature conservation areas due to the rich fauna and flora biodiversity found in these areas.[160] Government policy is increasingly prioritising the rehabilitation of these habitats for their biodiversity value and capacity to sequester carbon following the cessation of commercial peat extraction for electricity generation.[161,162]

Three plant extracts were selected for therapeutic exploration following screening of traditional folklore source material to identify plants historically used to treat gastrointestinal ailments. The plant extracts were subjected to in vitro testing, and in vivo testing using a dextran sulphate sodium-induced colitis mouse model. The plant extracts selected were: *Cardamine pratensis* (cuckoo flower), *Potentilla erecta* (tormentil), and *Erica tetralix* (cross-leaved heath). Each extract demonstrated acceptable cell viability in THP-1 cells, a human monocytic leukaemia cell line used as a model for studying monocyte/macrophage biology, by resazurin assay.[163] All extracts were tested at 100 mg/mL which corresponds to the highest tested concentration in vitro with acceptable cell viability. Further to this each extract showed inhibitory effects on inflammatory mediator production, with activity confirmed against comparator collections from the same species. This work was previously carried out by the NatPro Centre and remains unpublished. These findings provided the rationale for advancing these extracts into ex vivo explant testing.

We employed an explant model that has been validated in IBD and has the ability to capture the complex microenvironment of inflammatory colonic diseases allowing for drug discovery.[49,50]

4.3. Methods

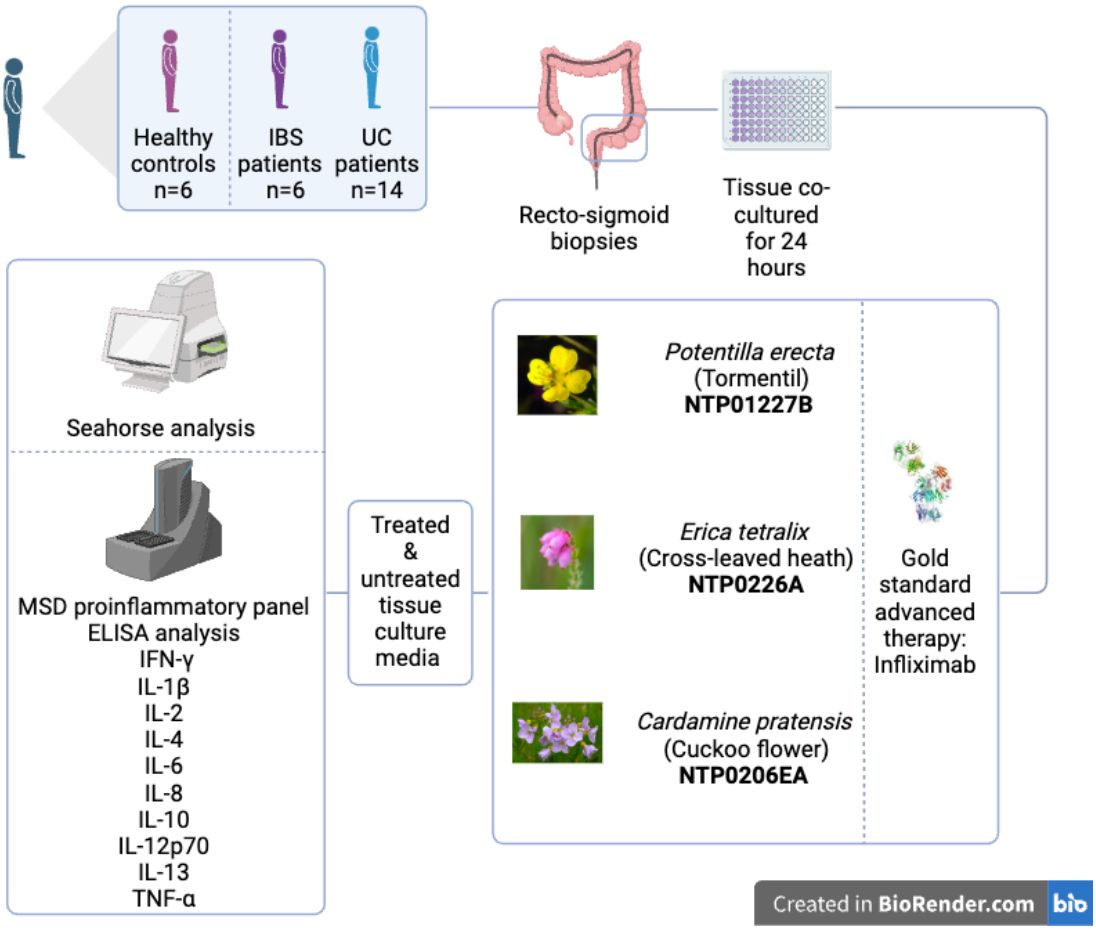


Figure 22. Experimental design characterising the changes in inflammatory and metabolic profiles following ex vivo explant treatment with infliximab, cuckoo flower, tormentil, and cross-leaved heath. HC, UC patients and IBS patients were recruited at endoscopy. Rectosigmoid biopsies were collected. Colonic tissue explants were generated, and co-cultured with drug or control. Seahorse Xfe24 analyser was used to generate metabolism profiles. MSD pro-inflammatory panel undergoing ELISA analysis was used to generate inflammatory profiles. Inflammatory markers included IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α. ECAR, extracellular acidification rate; ELISA, enzyme-linked immunosorbent assay; HC, healthy controls; IL, interleukin; IFN, interferon; IBS, irritable bowel syndrome; OCR, oxygen consumption rate; TNF, tumour necrosis factor; UC, ulcerative colitis.

4.3.1. Study population

A cohort was prospectively recruited at St James's Hospital, Dublin, following approval of the study by The St. James' Hospital / Tallaght University Hospital Joint Research Ethics Committee. Patients were recruited in three cohorts: healthy controls (HC), IBS patients and UC patients. HC were individuals without gastrointestinal (GI) symptoms who were undergoing endoscopy for polyp or family history surveillance, and had a colonoscopy without colitis. The diagnosis of IBS was made using established clinical, endoscopic and histological criteria[52]. Patients who had an increase of GI symptoms with a normal colonoscopy were included. UC patients undergoing endoscopic assessment for evaluation of disease activity, treatment response or for surveillance were included. The diagnosis of UC was made using established clinical, endoscopic and histological criteria[5,122]. Baseline demographics and information on medication exposure at the time of endoscopy were collected for each subject. An endoscopic Mayo subscore was documented for each included UC subject. UC in remission was defined as an endoscopic Mayo score of 0 or 1. Active UC was defined as an endoscopic Mayo score of 2 or 3. (**Table 11**) Endoscopies were performed by the treating GI team. The Endoscopist was solely responsible for grading disease severity.

4.3.2. Study endpoint definitions

The co-primary endpoints of this study were:

i) to determine whether ex vivo treatment of UC explants altered the inflammatory-metabolic profile; and

ii) to determine whether extracts from Cardamine pratensis (cuckoo flower), Potentilla erecta (tormentil), or Erica tetralix (cross-leaved heath) altered the inflammatory-metabolic profile of HC, IBS or UC colonic explants.

4.3.3. Ex vivo ulcerative colitis tissue explant culture

All patients undergoing colonoscopy had six study-related endoscopic biopsies taken. These biopsies were collected from the sigmoid colon approximately 20 cm from the anorectal margin, in the most severely inflamed area for UC patients. The endoscopic Mayo score from the sigmoid biopsy site was documented. Biopsies were placed in a standard specimen container which contained sterile gauze and saline. The specimen containers were immediately transported to the laboratory for processing following their collection. Colonic biopsies were then used to generate human colonic explants. Biopsies were washed with wash buffer (PBS (Corning, the USA), 1% Pen/Strep (Gibco, Themofisher, the USA), 1% Fungizone (Sigma-Aldrich, the USA), 0.1% Gentamicin (Lonza, Switzerland)) and then cultured in a 24 well plate with media (M199 (Gibco, Themofisher, the USA), 10% FBS (Lonza, Switzerland), 1% Pen/Strep, 1% Fungizone, 0.1% Gentamicin, 1 ug/ml Insulin (Lonza, Switzerland)). For each study participant, six biopsies were co-cultured with two control vehicles and four treatments. The two control treatments were 0.1% dimethyl sulfoxide (DMSO) [control vehicle for plant extracts], 10% immunoglobulin G (IgG) [control vehicle for infliximab]. The four distinct treatments were 10% infliximab (IFX), 100 µg/mL of an ethyl acetate extract of the aerial parts of cuckoo flower, 100 µg/mL of a methanol extract of the root of tormentil and 100 µg/mL of a methanol extract of the aerial parts of cross-leaved

heath. Biopsies were incubated for 24 hours at 37 °C with 95% O2/5% CO2 humidified atmosphere to generate human explant-CM. The explant model described in this report has been previously optimised by our group, with assays confirming tissue viability for up to 72 hours[49,123].

4.3.4. Determination of energy metabolism of explant-CM

In the last hour of culture, colonic biopsies and explant-CM were transferred to an islet capture microplate with capture screens (Agilent Technologies, Santa Clara, California, USA) and incubated in a non-CO2 incubator at 37 °C (Whitley, West Yorkshire, UK) prior to analysis. Seahorse Xfe24 analyser was used to assess metabolic profiles in colonic explants (Agilent Technologies, CA, USA). Following a 12 minute equilibrate step, three basal measurements of OCR (Oxygen Consumption Rate) and ECAR (Extracellular Acidification Rate) were taken over 24 minutes consisting of three repeats of the following sequence "mix (3 minutes)/wait (2 minutes)/measurement (3 minutes)" to establish basal respiration. Explant-CM was extracted in a sterile environment and tissue was snap frozen. All samples were then stored at −80 °C for further processing.

4.3.5. Determination of secreted protein concentrations in explant-CM

Explant-CM secreted proteins were quantified using a V-PLEX Proinflammatory Panel enzyme-linked immunosorbent assay (ELISA) kit (Meso Scale Diagnostics, the USA). These assays quantified the secretions of the following ten proteins: IFN-γ, IL-

1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α. All assays were performed according to manufacturer protocol. All secreted protein concentrations were normalised to total protein content of the biopsy tissue specimen by using a BCA assay (Pierce, the USA) as per manufacturers recommendations.

4.3.6. Extraction and quantification of biopsy protein levels

Biopsy tissue specimens were added to cryovials with one metal bead and 200ul RIPA buffer supplemented with EDTA-free protease inhibitor cocktail (Sigma-Aldrich, the USA) and phosphatase inhibitor (Roche, Switzerland). Biopsies were homogenised at 25 Hz for 2 minutes in a tissue lyser (Qiagen, Germany). Samples were transferred to clean Eppendorf tubes and centrifuged for 20 minutes at 1800 rcf at 4 °C. 10ul of each sample was added to 96 well plate in triplicates (Thermo Pierce BCA microassay, the USA) with 200ul of working reagent. The plate was incubated for 30 minutes at 37 °C in a humidified CO2 incubator, and absorbance was read at 562 nm to quantify the total protein content of the sample.

4.3.7. Statistical analysis

Baseline demographic and clinical data were presented as median and range for continuous variables and frequencies and percentages for categorical variables. The Mann–Whitney *U* test was used to compare continuous variables between groups by endoscopic remission and biologic therapy status. The Kruskal-Wallis test was used to compare difference in continuous variables between groups for age. The

Fisher's exact test was used to compare frequency data. Wilcoxon testing was employed to test for differences between paired variables. Principal Component Analysis (PCA) was performed using the prcomp function in R. Prior to conducting PCA, the data were scaled. A significance level of 0.05 was used in all analysis and all p-values. Statistical analysis was performed using GraphPad Prism 9.5 (GraphPad Software, California, United States) and R Core Team (2023). _R: A Language and Environment for Statistical Computing_. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.

4.4. Results

4.4.1. Baseline characteristics

Twenty-four patients were recruited cross three cohorts: six HC, six IBS subjects and fourteen subjects with UC. Baseline characteristics of the study cohort were similar across all groups. The median age at recruitment of the overall cohort was 52 years (IQR 43.5 – 61.25 years). The median age of subjects with UC at recruitment was lower than the HC and IBS cohorts (p=0.02). 62% of the overall cohort were male (n=16). There were no differences between cohorts for sex (p=0.27). 57% (n=8) of the UC cohort had active disease with 43% (n=6) being in endoscopic remission. 50% (n=7) were currently on a biologic and 36% (n=5) had prior anti-TNF exposure. Two patients were on an anti-TNF therapy at time of endoscopy. The majority of UC patients had left sided disease (64%, n=9). (**Table 14, Table 15**).

	Control (n=6)	IBS (n=6)	UC (n=14)			P value
Age at scope (median, IQR)	61.5 years (53.7 - 71)	56 years (42.3 – 67.5)	49 years (37 - 53)			**0.02**
Male sex (%)	5 (83)	2 (33)	6 (43)			0.27
Disease duration (median, IQR)			11.2 years (1.8 – 27.8)			
Disease extent (%)			Proctitis		2 (14)	
			Left-sided		9 (64)	
			Pan-colitis		3 (21)	
Previous Anti-TNF therapy (%)			5 (36)			
Current advanced therapy (%)			7 (50)			
Current Anti-TNF therapy (%)			2 (14)			
Endoscopic Mayo score (%)			0	4 (29)		
			1	2 (14)		
			2	6 (43)		
			3	2 (14)		

Table 14. Baseline Demographics of healthy controls, IBS and overall UC cohorts. Anti-TNF, anti-tumour necrosis factor; eMayo, endoscopic Mayo, IBS, irritable bowel syndrome; IQR, interquartile range; UC, ulcerative colitis.

		UC in remission (n=6)	Active UC (n=8)	P value
Age at scope (median, IQR)		50.5 years (43.5 - 54)	44.5 years (27.1 – 52.8)	0.33
Male sex (%)		2 (33)	4 (50)	0.62
Disease duration (median, IQR)		16.2 years (3.8 – 20.3)	9.6 years (5.8 – 15.8)	0.66
Disease extent (%)	Proctitis	1 (17)	1 (13)	
	Left-sided	4 (67)	5 (63)	
	Pan-colitis	1 (17)	2 (25)	
Current advanced therapy (%)		2 (53)	5 (63)	0.59
Current Anti-TNF therapy (%)		0 (0)	2 (25)	
Endoscopic Mayo score (%)	eMayp 0	4 (67)		
	eMayo 1	2 (33)		
	eMayo 2		6 (75)	
	eMayo 3		2 (25)	

Table 15. Baseline Demographics of UC in remission cohort and UC with active disease cohort. Anti-TNF, anti-tumour necrosis factor; eMayo, endoscopic Mayo, IBS, irritable bowel syndrome; IQR, interquartile range;

4.4.2. Changes in inflammatory mediator secretion and energy metabolism markers following ex vivo infliximab treatment in UC

In the overall UC cohort the median TNF-α secretion levels reduced from a median of 2.54 pg/ml (IQR, 1.67 - 5.56) in the control to 1.69 pg/ml (0.9 – 2.54) in the ex vivo infliximab treated group (p=0.0494). Similar reductions in IL-12p70 (0.299 pg/ml (0.12 – 0.50) to 0.052 pg/ml (0.04 – 0.196) p=0.0245) and IL-4 (0.07 (0.0491 - 0.163) to 0.054 (0.036 – 0.091) p=0.0419) were seen. No changes in energy metabolism markers were seen in the UC cohort following ex vivo infliximab treatment. (**Figure 23**)

Analysis of the UC group subdivided by endoscopic remission status showed no changes in inflammatory protein secretion or metabolism markers of the UC in remission group following ex vivo infliximab treatment. In the UC with active disease cohort TNF-α (median, (IQR), 3.45 pg/ml (2.46 – 7.6) to 1 pg/ml (0.58 – 2.3), p=0.008) and IL-4 (0.1 pg/ml (0.06 – 0.2) to 0.06 pg/ml (0.05 - 0.16), p=0.02) secretion reduced following treatment. (**Figure 24**) No change in secretion of the following proteins was observed following ex-vivo infliximab treatment in the overall cohort and when divided by disease activity: IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, and IL-13. Subgroup analysis was performed excluding those exposed to anti-TNF therapy (either previously or currently). No difference in inflammatory protein secretion or metabolism markers was observed following ex vivo treatment with IFX however the sample size was small (n = 7). Additionally there was no available data on mechanism of anti-TNF failure.

Principal component analysis (PCA) shows that following ex vivo infliximab treatment there is no reduction in separation of clustering of the UC cohort from HC

or IBS cohorts, compared to their untreated inflammatory-metabolic profiles. (**Figure 30 A & B**)

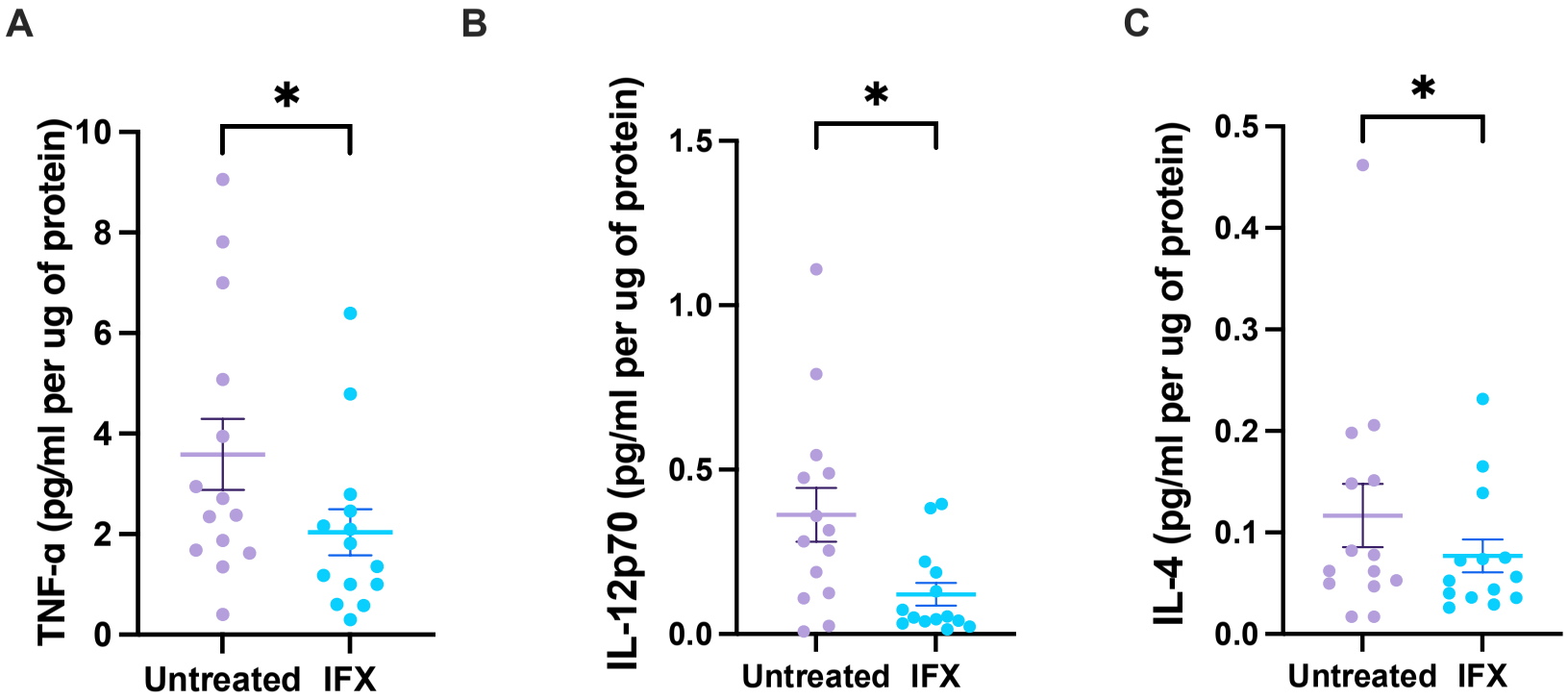


Figure 23. Changes in inflammatory proteins following ex vivo infliximab treatment of UC explants. In overall UC cohort (A) TNF-α, (B) IL-12p70 and (C) IL-4 secretion are significantly reduced following infliximab treatment (p=0.049, p=0.02, and p=0.04 respectively). Graph shows Mann-Whitney U test, scatter plot (median ± IQR). Statistically significant P value of <0.05, denoted with *. IFX, infliximab; IL, interleukin; TNF, tumour necrosis factor.

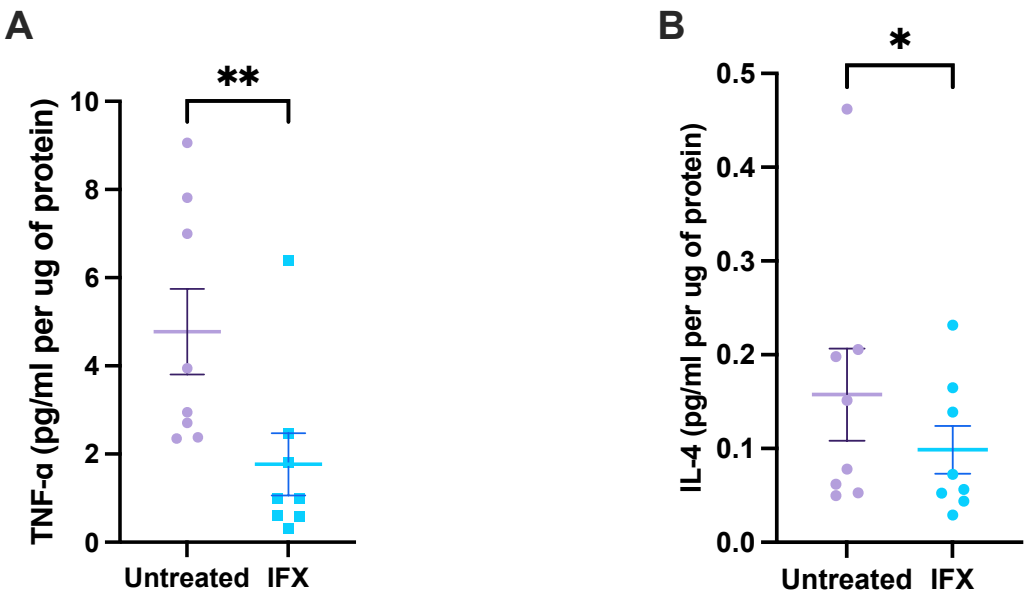


Figure 24. Changes in inflammatory proteins following ex vivo infliximab treatment of explants from UC with active disease cohort. In the UC with active disease cohort (A) TNF-α, (B) IL-4 secretion are significantly reduced following infliximab treatment (p<0.01 and p=0.02, respectively). Graph shows Mann-Whitney U test, scatter plot (median ± IQR). Statistically significant P value of <0.05, denoted with * <0.01, denoted with **. IFX, infliximab; IL, interleukin; TNF, tumour necrosis factor.

4.4.3. Changes in inflammatory mediator secretion and energy metabolism markers following ex vivo cuckoo flower treatment

In the HC cohort IL-1β was significantly reduced compared to untreated control (median (IQR), 0.9957 pg/ml (0.8 – 1.63) following ex vivo treatment with cuckoo flower extract (0.52 pg/ml (0.4765 – 0.8058)) (p=0.0312). No changes were seen in energy metabolism markers following ex vivo cuckoo flower extract treatment in the HC cohort. (**Figure 25**)

In the overall UC cohort IFN-γ (0.4 pg/ml (0.067 – 11.14) to 0.127 pg/ml (0.041 – 1.135), p=0.0134) and IL-10 (0.237 pg/ml (0.145 – 1.137) to 0.159 pg/ml (0.076 – 0.4668), p=0.0166) secretion significantly reduced following treatment with cuckoo flower extract. Analysis of the UC group subdivided by endoscopic remission status showed no changes in inflammatory protein secretion or metabolism markers of the UC in remission group following ex vivo cuckoo flower treatment. Only IFN-γ reduced significantly in the UC with active disease cohort following treatment (4.2 pg/ml (0.27 – 4.19) to 0.96 pg/ml (0.12 – 2.9), p=0.04). (**Figure 25, Figure 26**)

In the IBS cohort the OCR was significantly reduced following ex vivo cuckoo flower extract treatment (46.72 pmol/min (30.58 – 627.5)) compared to the untreated control (211.5 pmol/min (75.92 - 796.7)) (p=0.0312). No changes were seen in inflammatory mediator secretion following ex vivo cuckoo flower extract treatment in the IBS cohort. (**Figure 25**)

The PCA plots show that following ex vivo cuckoo flower extract treatment there is reduced separation between the HC and IBS cohorts, as well as between HC and UC cohorts, compared to their untreated inflammatory-metabolic profiles. (**Figure 30 C & D**)

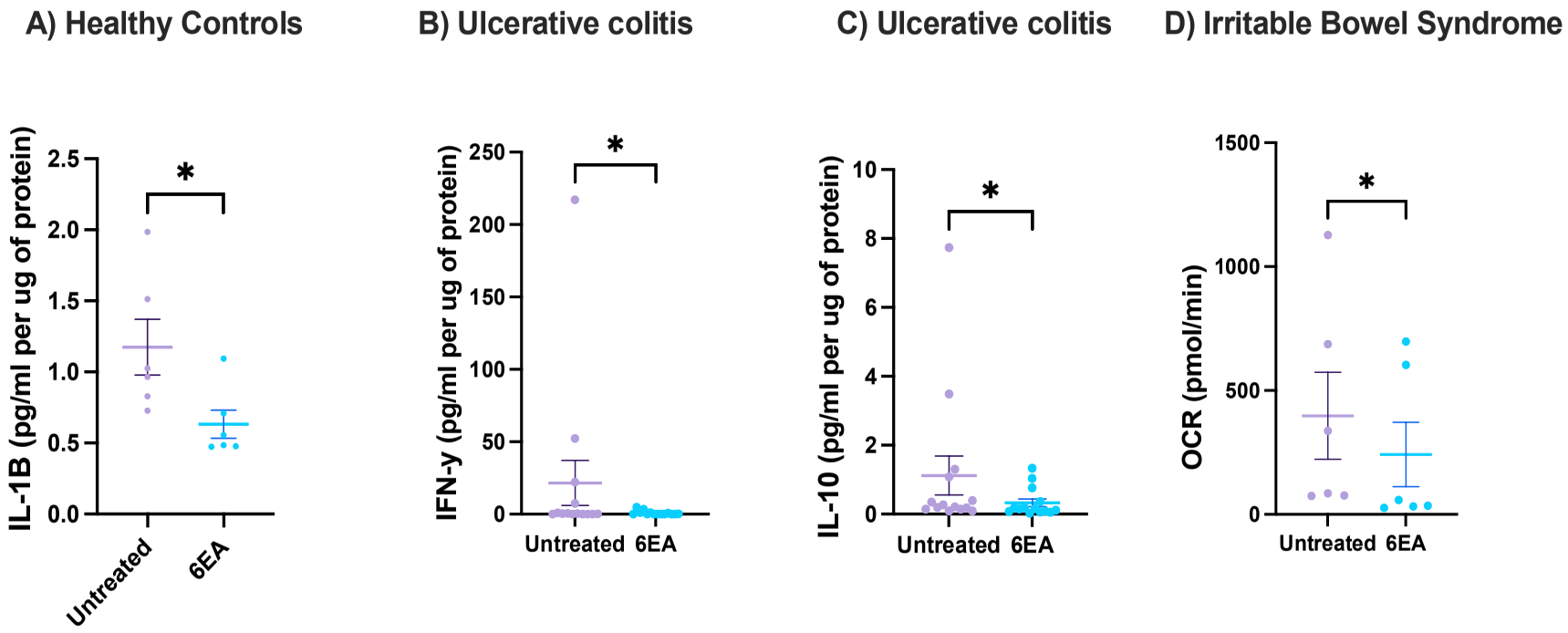


Figure 25. Changes in metabolism and inflammatory profiles following ex vivo treatment with cuckoo flower extract (6EA). A) IL-1β is significantly reduced post treatment in healthy controls (p=0.03). B & C) In UC cohort IFN-γ and IL-10 secretion are significantly reduced following treatment with cuckoo flower extract (p=0.01, and p=0.02 respectively). D) In IBS cohort OCR was significantly reduced following cuckoo flower extract treatment (p=0.03). Graph shows Mann-Whitney U test, scatter plot (median ± IQR). Statistically significant P value of <0.05, denoted with *. IFN, interferon; IL, interleukin; 6EA, cuckoo flower extract.

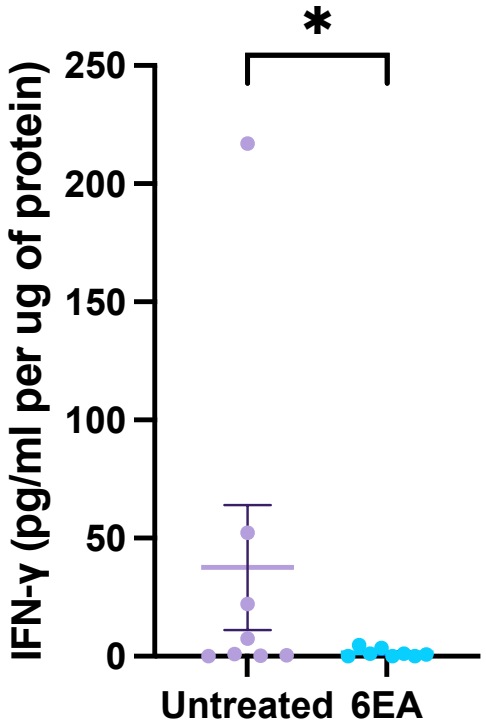


Figure 26. Changes in inflammatory proteins following ex vivo cuckoo flower extract (6EA) treatment of explants from UC with active disease cohort. In the UC with active disease cohort IFN-γ secretion significantly reduced following treatment with cuckoo flower extract (p=0.04). Graph shows Mann-Whitney U test, scatter plot (median ± IQR). Statistically significant P value of <0.05, denoted with *. IFN, interferon; 6EA, cuckoo flower extract.

4.4.4. Changes in inflammatory mediator secretion and energy metabolism markers following ex vivo tormentil treatment

No changes were seen in inflammatory mediator secretion and energy metabolism markers following ex vivo tormentil extract treatment in the HC cohort.

In the overall UC cohort IL-10 secretion was significantly reduced following treatment with tormentil extract (0.2372 pg/ml (0.145 – 1.137) to 0.1676 pg/ml (0.05783 – 0.3105), p=0.002). No changes in energy metabolism markers were seen in the UC cohort following ex vivo tormentil extract treatment. Analysis of the UC group subdivided by endoscopic remission status showed no significant changes in inflammatory protein secretion or metabolism markers of both the UC in remission group and the UC with active disease group following ex vivo tormentil treatment. (**Figure 27**)

In the IBS cohort IL-2 (median (IQR), 0.07846 pg/ml (0.04243 – 0.3764) to 0.0635 pg/ml (0.01142 – 0.09905), p=0.0312) and IL-10 (0.0936 pg/ml (0.05883 - 0.5095) to 0.03681 pg/ml (0.0128 – 0.1428), p=0.0312) were significantly reduced following ex vivo treatment with tormentil extract. No changes in energy metabolism markers were seen in the IBS cohort following ex vivo tormentil extract treatment. (**Figure 27**)

PCA plots shows that following ex vivo tormentil extract treatment there is reduced separation between the HC and IBS cohorts, as well as between HC and UC cohorts, compared to their untreated inflammatory-metabolic profiles. **(Figure 30 C & E)**

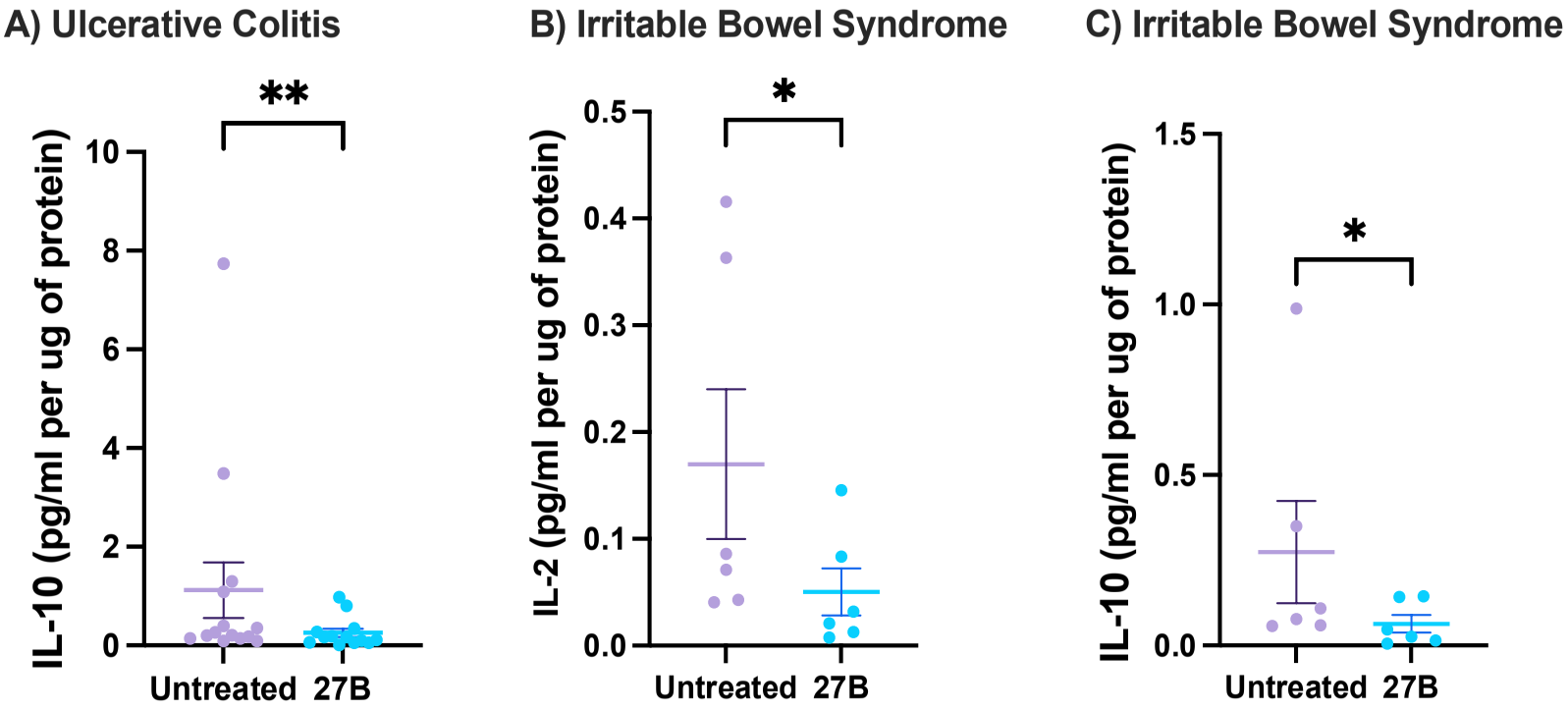


Figure 27. Changes in inflammatory profile following ex vivo treatment with tormentil extract (27B). A) In the UC cohort IL-10 secretion is significantly reduced following treatment with tormentil extract (p=0.002 B & C) In the IBS cohort IL-2 and IL-10 are significantly reduced following ex vivo treatment with tormentil extract (p=0.03, and p=0.03 respectively). Graph shows Mann-Whitney U test, scatter plot (median ± IQR). Statistically significant P value of <0.05, denoted with *. IL, interleukin; 27B, tormentil extract.

4.4.5. Changes in inflammatory mediator secretion and energy metabolism markers following ex vivo cross-leaved heath treatment

No changes were seen in inflammatory mediator secretion and energy metabolism markers following ex vivo cross-leaved heath extract treatment in the HC cohort.

In the overall UC cohort TNF-α secretion was significantly increased following treatment with cross-leaved heath extract (median, (IQR), 2.479 pg/ml (1.391 – 4.651) to 3.86 pg/ml (2.254 – 10.95), p=0.0419). No changes in energy metabolism markers were seen in the UC cohort following ex vivo cross-leaved heath extract treatment. Analysis of the UC group subdivided by endoscopic remission status showed no significant changes in inflammatory protein secretion or metabolism markers of the UC in remission group following ex vivo cross-leaved heath extract treatment. In the UC with active disease cohort TNF-α secretion significantly increased following treatment (3.42 pg/ml (1.1 – 4.9) to 9.6 pg/ml (4.6 – 12.4), p=0.02). (**Figure 28**, **Figure 29**)

In the IBS cohort IL-4 (median (IQR), 0.031 pg/ml (0.0157 – 0.0944) to 0.013 pg/ml (0.005 – 0.033), p=0.0312) and IL-6 (269.6 pg/ml (101.6 – 5446) to 59.01 pg/ml (24.05 – 537.1), p=0.0312) are significantly reduced following ex vivo treatment with cross-leaved heath extract. No changes in energy metabolism markers were seen in the IBS cohort following ex vivo cross-leaved heath extract treatment. (**Figure 28**)

PCA plots shows that following ex vivo cross-leaved heath extract treatment there is reduced separation between the HC and IBS cohorts, as well as between HC and

UC cohorts, compared to their untreated inflammatory-metabolic profiles. (**Figure 30 C & F**)

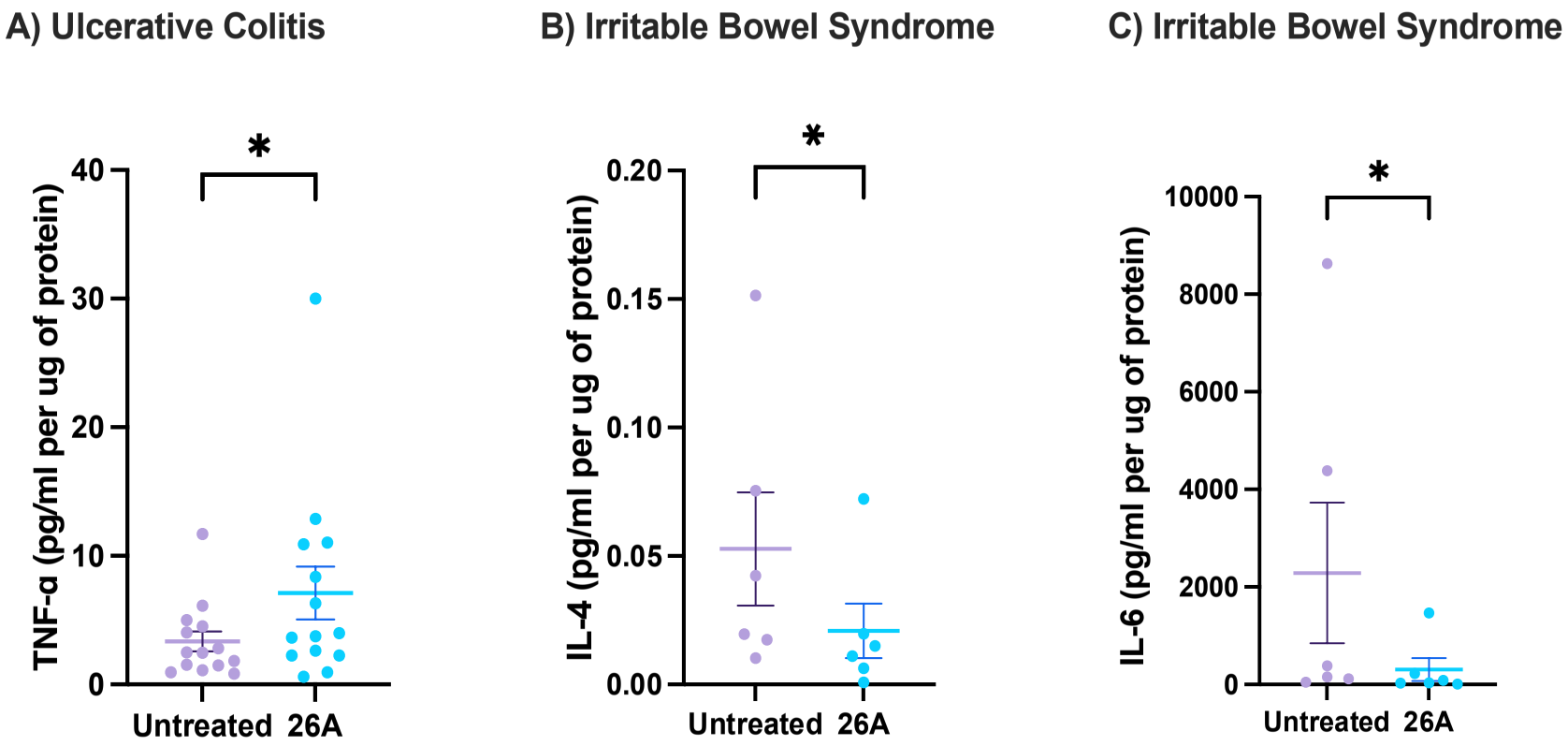


Figure 28. Changes in metabolism and inflammatory profiles following ex vivo treatment with cross-leaved heath extract (26A). A) In the overall UC cohort TNF-α secretion is significantly increased following treatment with cross-leaved heath extract (p=0.04). B & C) In the IBS cohort IL-4 and IL-6 are significantly reduced following ex vivo treatment with cross-leaved heath extract (p=0.03, and p=0.03 respectively). Graph shows Mann-Whitney U test, scatter plot (median ± IQR). Statistically significant P value of <0.05, denoted with *.IL, interleukin; TNF-α, tumour necrosis factor-alpha; 26A, cross-leaved heath extract.

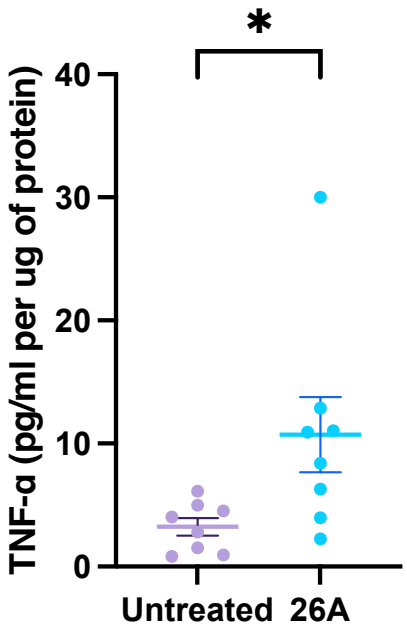


Figure 29. Changes in inflammatory proteins following ex vivo cross-leaved heath extract (26A) treatment of explants from UC with active disease cohort. In the UC with active disease cohort TNF-α secretion is significantly increased following treatment with cross-leaved heath extract (p=0.02). Graph shows Mann-Whitney U test, scatter plot (median ± IQR). Statistically significant P value of <0.05, denoted with *. TNF-α, tumour necrosis factor-alpha; 26A, cross-leaved heath extract.

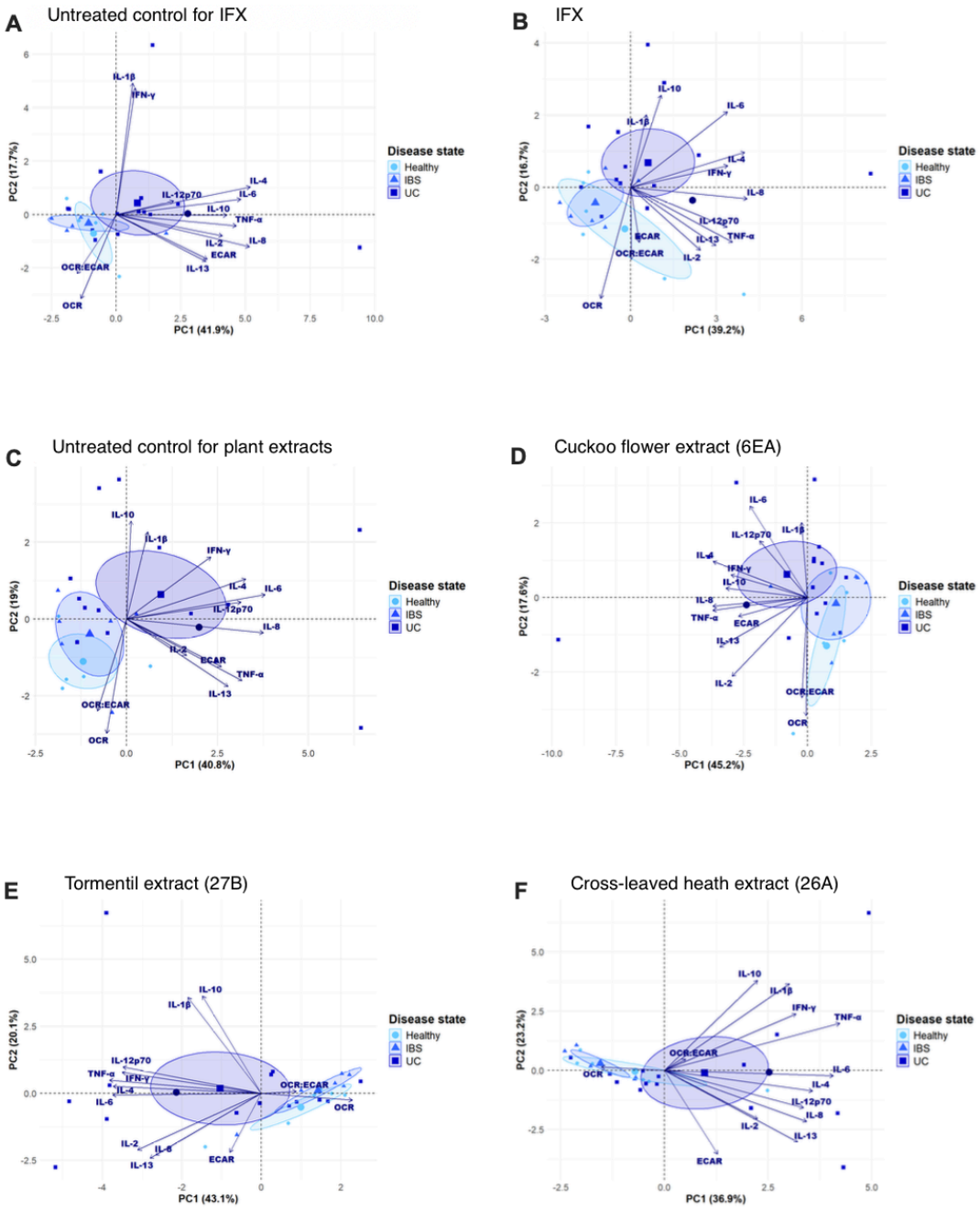


Figure 30. Principle Component Analysis showing changes in inflammatory-metabolic profiles following ex vivo treatment with infliximab or natural products. IFX, infliximab; PC, principal component; 6E, cuckoo flower extract; 26A, cross-leaved heath extract; 27B, tormentil extract. A) Untreated IgG control vehicle to compare with; B) infliximab. C) Untreated DMSO control vehicle to compare with; D) cuckoo flower extract (6EA), E) tormentil extract (27B) and F) cross-leaved heath extract (26A). Figures D to F show reduced separation between both healthy controls and IBS tissue, and healthy controls and UC tissues.

4.5. Discussion

In this report we present a pilot study utilising human ex vivo explants to evaluate the effects of infliximab on the UC inflammatory and metabolic profile, and of three natural extracts on the inflammatory and metabolic profile of the healthy, IBS and UC microenvironment.

Infliximab treatment significantly reduced TNF-α, IL-12p70 and IL-4 secretion in explants from the overall UC cohort. This validates the experimental model by confirming the established mechanism of action of infliximab, and demonstrating infliximab's immune-modulating properties.[164] Cuckoo flower treatment reduced IL-1β secretion in the HC cohort, reduced IFN-γ and IL-10 secretion in the overall UC cohort, and reduced oxidative phosphorylation in IBS patients. Tormentil treatment led to reduced IL-10 secretion in the overall UC cohort and reduced IL-2 and IL-10 secretion in the IBS cohort. Cross-leaved heath extract reduced IL-4 and IL-6 secretion in IBS tissue but increased TNF-α in the overall UC cohort.

Infliximab is an anti-TNFα therapy licenced to treat patients with moderately to severely active UC with an inadequate response to conventional therapy.[165] It is a monoclonal antibody that binds TNF-α. It then neutralizes TNF-α and forms an immune complex. It has emerged that, unlike in rheumatoid arthritis, the role of anti-TNF therapy in IBD is not solely due to this interaction. Our work shows a reduction in IL-12p70 secretion following infliximab treatment. This finding supports previous research suggesting that the immune complex interacts, via the Fc region on

infliximab, with Fcγ receptors on macrophages. This results in blockade of IL-12/IL-23 secretion.[88,164,166,167]

TNF-α inhibition is mainly associated with reduction of T helper (Th) 1 responses. Interestingly, IL-4 secretion reduced following ex vivo infliximab treatment. IL-4 is a driver of Th2 cell differentiation and mediates allergic responses. IL-4 induces a phase of chronic colitis in IL-10 deficient mice, suggesting it has pro-inflammatory properties also.[168,169] In the previous chapter, using the same experimental model, we found IL-4 levels to be elevated in active UC. There are inconsistent reports regarding the effects of infliximab on IL-4 secretion. Mucosal biopsies before and 4 weeks following in vivo infliximab treatment showed unchanged IL-4 levels in a 2009 study of 32 patients and 19 controls.[49,170,171] Inhibition of TNF-α may reduce IL-4 secretion indirectly as a consequence of broader immune and cytokine network changes during treatment. These findings suggest a complementary mechanism of action of infliximab.

Existing clinical data suggests potential efficacy of tormentil in treating UC. A small, uncontrolled study involving 16 patients with UC, tested tormentil as a treatment for active disease. The study had notable limitations, such as the lack of objective outcome measures. However, reductions in both the clinical activity index scores and CRP levels during the treatment period, followed by increases during the washout phase, were seen. Notably were mild and included upper abdominal discomfort and heartburn. This suggests therapeutic potential of tormentil for inducing remission in UC. The authors proposed that tannins within tormentil may

modulate the microbiome and promote mucosal healing, though the exact mechanism of action remains unclear.[172]

In contrast, our ex vivo findings revealed that tormentil treatment reduced IL-10 secretion in UC explants. IL-10 is a critical anti-inflammatory cytokine responsible for downregulating the Th1 pathway and maintaining gut homeostasis.[173] Reducing IL-10 may prolong intestinal inflammation, making this an undesirable therapeutic outcome. Similarly, in IBS, IL-10 secretion was reduced alongside IL-2. IL-2 has a dual role in the immune system: as a potent immune activator and as a regulator of immune tolerance via T regulatory cells. While little is known about IL-2 in IBS, low-dose IL-2 has shown promising results in UC treatment [174]. Our findings do not support the use of tormentil for treating UC or IBS.

Ex vivo treatment with cuckoo flower demonstrated differing effects across the three groups tested, highlighting its complex inflammatory and metabolic modulatory properties. In healthy controls, cuckoo flower reduced IL-1β, a pro-inflammatory cytokine known to impair gut barrier function and contribute to UC pathology [175]. In UC explants, however, cuckoo flower treatment led to a simultaneous reduction in IFN-γ and IL-10 secretion, suggesting opposing effects. IFN-γ, primarily produced by mucosal T cells and type 1 innate lymphoid cells, plays a pivotal role in initiating mucosal inflammation but does not sustain it. By contrast, IL-10 functions as a critical anti-inflammatory cytokine, reducing mucosal effector T cell activation and preserving epithelial integrity through inhibition of epithelial apoptosis [168]. This dual effect is unlikely to provide therapeutic benefit for UC patients, as reducing IL-10 may prolong inflammation. A more targeted approach, considering specific inflammation subtypes and disease stages, may be required to fully understand the therapeutic potential of cuckoo flower for UC.

In IBS, cuckoo flower treatment reduced oxidative phosphorylation. This suggests cuckoo flower may cause mitochondrial dysfunction. This would be an undesirable effect, given the integral role mitochondria play in energy production, gut barrier maintenance, and immune regulation through their influence on innate and adaptive responses. [73,176]

In this study, we observed a significant reduction in IL-4 and IL-6 secretion in the IBS subgroup following ex vivo treatment with cross-leaved heath extract, with no effects noted in healthy controls. Activated mast cells and their mediators are found in high abundance within the IBS microenvironment, suggesting their role in disease evolution. [58] IL-4 plays a central role in mast cell activation, a key feature of non-classical food allergies frequently reported in IBS patients.[54] While IL-4 is classically involved in IgE-mediated allergies, it may also indirectly contribute to non-IgE-mediated allergies by reducing mast cell sensitisation. IL-4 inhibition has shown significant efficacy in treating allergic and atopic conditions, as demonstrated by Dupilumab, an IL-4 inhibitor currently used to treat severe asthma and atopic dermatitis. [177,178]

IL-6, on the other hand, is a pro-inflammatory cytokine that promotes Th17 differentiation and has been implicated in gut inflammation. Elevated circulating IL-6 levels have been observed in IBS-D patients, further linking IL-6 to IBS pathogenesis [179]. Olamkicept, a selective IL-6 trans-signalling inhibitor, has demonstrated promising results in ulcerative colitis.[128,129] Given the role of IL-4 on mast cell activation and IL-6 in promoting inflammation, targeting both allergy and inflammation presents an attractive potential therapeutic approach for IBS. Our

findings support further exploration of cross-leaved heath extract as a treatment for IBS.

The discrepancy between the clinical improvements reported in the tormentil trial and the results of our ex vivo testing highlights the limitations of ex vivo models in fully capturing real-world biological complexity. Although ex vivo studies offer valuable mechanistic insights, they cannot fully replicate the dynamic interactions within the human gut. However, as with all ex vivo testing, these results must be interpreted with caution, and further research using integrative models is warranted to better understand the therapeutic potential of these extracts in gastrointestinal diseases.

While these findings confirm cuckoo flower treatment ex vivo has inflammatory and metabolic properties, the contrasting and undesirable effects observed underscore the challenges of translating such findings into viable therapies. Further investigation is needed to clarify how these interactions might manifest in vivo and whether more refined, disease-specific approaches could mitigate these limitations. Notably, no human studies have evaluated the use of cuckoo flower as a medicinal or herbal preparation to assess its in vivo safety and tolerability, and its side-effect profile therefore remains unknown.

We propose cross-leaved heath extract to be a suitable candidate for further analysis with regards to its therapeutic potential in IBS, given the potential for reduction in mast cell activation and IL-6 inhibition. Future research should focus on its use in IBS-diarrhoea, and post infectious IBS. Further avenues of therapeutic exploration should be considered including atopy and food-intolerance. Combined suppression

of IL-4 and IL-6 may increase susceptibility to infection. Consideration regarding the mode of treatment delivery and dosing should be integral to further investigation to limit the potential immunosuppressive effects. Given the increase in TNF-α levels following treatment of the UC cohort, a cautious approach must be taken with respect to its therapeutic potential in UC or IBS/IBD overlap syndromes. Again, no human studies have assessed its in vivo side-effect profile; further in vivo studies are required to evaluate its safety and tolerability.

The major limitations of this study were small sample size and heterogeneity in the patient cohort. Half the UC cohort were currently on an advanced therapy. It is unclear what effects the current or previous use of these therapies might exert on the IBD microenvironment. To help overcome the small sample size we analysed the effects of the therapies on the UC cohort in total, as well as by endoscopic remission status. There was limited clinical data available pertaining to the IBS cohort. There was no phenotypic subtype or symptom scores available for this cohort. Further research should focus on differences in treatment outcomes between IBS-diarrhoea and post-infectious IBS, as these subtypes have the strongest evidence of immune dysregulation.[58,180]

Notably, our study further supports the use of patient derived explants for drug discovery in IBD. Explants have the ability to recapitulate disease biology more precisely and reflect the heterogeneity of patient populations more accurately than traditional IBD disease models. Additionally, they are a more cost effective and sustainable mechanism of drug discovery compared to traditional methods of drug

discovery, and avoid the ethical and welfare concerns frequently associated with the use of animal models. [48,49,181]

Natural extracts have more complex structures when compared to synthetic compounds, which may be beneficial for drug delivery and efficacy. However, there may be non-bioactive substances within the extract requiring further refinement prior to drug development. Following this, compounds will need to undergo rigorous safety testing given the pleotropic effects we observed. Natural extracts are also known to affect the gut microbiome. Future work should interrogate how these products affect the host microbial community, which could reveal further mechanisms of action.[87] A further benefit of cultivating and repurposing plants in this context is the potential for an alternative means for landowners to derive an economic return from the management of peatlands. This may be of particular significance at a time when national and European Union policy is increasingly prioritising both a shift towards a more circular economy, and a move away from peat harvesting and intensive agricultural activity on peatlands towards restoration of peatlands for biodiversity and carbon sequestration benefits.[161,162]

4.6. Conclusion

Our study, exploratory in nature, revealed additional effects of infliximab therapy on UC mucosa and supports the selection of cross-leaved heath for further investigation as a treatment of IBS. Additionally consideration of its use in non-gastrointestinal disorders such as allergy should be considered.

4.7. Summary of findings of chapter 4

In this chapter, we assessed whether the inflammatory-metabolic profiles of HC, UC patients' and IBS patients' colonic explants were modifiable by an existing therapy (infliximab) and three novel plant extracts (cuckoo flower, tormentil, and cross-leaved heath).

We reported that ex vivo treatment of the UC explant with infliximab reduced TNF-α, IL-12p70 and IL-4 secretion. This finding supports previous research suggesting that the immune complex formed by the infliximab-TNF-α interaction, causes blockade of IL-12/IL-23 secretion.[88,164,166,167] Reduction in IL-4 secretion following infliximab therapy has not been widely reported previously. This likely reflects broader immune and cytokine network changes during treatment.

The three natural extracts had pleotropic effects on the colonic tissue microenvironment, and these effects differed across disease class. Cross-leaved heath extract reduced both IL-4 and IL-6 in the IBS explant. Inhibition of the IL-4 pathway has been shown to reduce mast cell activation. Mast cell activation has been implicated in the pathogenesis of IBS.[54,182] Circulating levels of IL-6 have been found to be raised in IBS patients, specifically in those with IBS diarrhoea predominant disease.[179] As such, cross-leaved heath has been recommended for further therapeutic exploration. Consideration should also be given to its potential application to non-GI disorders, including food allergy, allergic skin conditions, and atopy.

These findings contribute to our understanding of the complex interplay in the colonic microenvironment and select a potential candidate natural extract for therapeutic exploration.

Chapter 5: Discussion

5.1. Aims of Thesis

I. To quantify the effectiveness, safety and cost of current treatment strategies used in complex IBD.

II. To investigate whether a specific inflammatory and metabolic profile is associated with UC or IBS.

III. To investigate whether infliximab and/or novel natural plant extracts alter the inflammatory and metabolic profiles in UC and IBS.

5.2. Study Application

At its core, this thesis sets out to evaluate whether cuckoo flower, tormentil, and cross-leaved heath have therapeutic potential in UC or IBS. The findings of this thesis do not support this hypothesis in UC. However, cross-leaved heath extract has demonstrated therapeutic potential in IBS and warrants further investigation.

5.3. Summary of Findings and Discussion

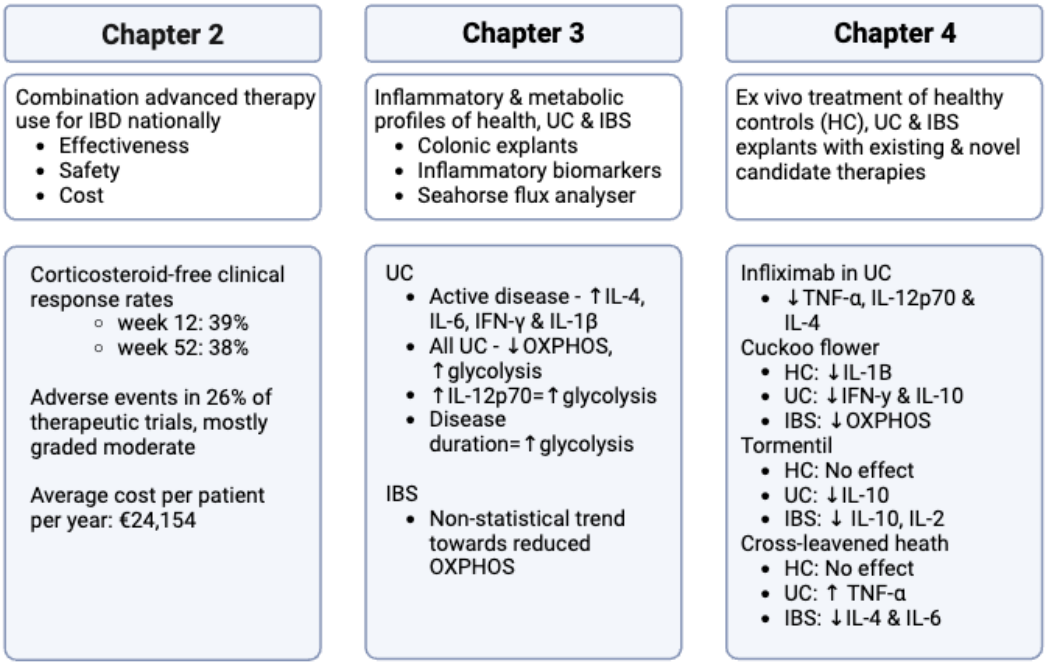


Figure 31. Schematic overview of thesis findings. HC, healthy controls; IBD; inflammatory bowel disease; IBS, irritable bowel syndrome; IFN, interferon; IL, interleukin; OXPHOS, oxidative phosphorylation; TNF, tumour necrosis factor; UC, ulcerative colitis.

Treatment	Marker	HC	IBS	UC (Overall)	UC Remission	UC Active
Baseline (untreated)	TNF-α	–	–		–	–
	IFN-γ	–	–		–	↑
	IL-1β	–	–		–	↑
	IL-2	–	–		–	–
	IL-4	–	–		–	↑
	IL-6	–	–		–	↑
	IL-8	–	–		–	–
	IL-10	–	–		–	–
	IL-12p70	–	–		–	–
	IL-13	–	–		–	–
	OCR	–	–		↓	↓
	ECAR	–	–		–	–
	OCR:ECAR	–	–		↓	↓
Infliximab (IFX)	TNF-α			↓	–	↓
	IFN-γ			–	–	–
	IL-1β			–	–	–
	IL-2			–	–	–
	IL-4			↓	–	↓
	IL-6			–	–	–
	IL-8			–	–	–
	IL-10			–	–	–
	IL-12p70			↓	–	–
	IL-13			–	–	–
	OCR			–	–	–
	ECAR			–	–	–
	OCR:ECAR			–	–	–
Cuckoo flower	TNF-α	–	–	–	–	–
(*Cardamine pratensis*)	IFN-γ	–	–	↓	–	↓
[6EA]	IL-1β	↓	–	–	–	–
	IL-2	–	–	–	–	–
	IL-4	–	–	–	–	–
	IL-6	–	–	–	–	–
	IL-8	–	–	–	–	–
	IL-10	–	–	↓	–	–
	IL-12p70	–	–	–	–	–
	IL-13	–	–	–	–	–
	OCR	–	↓	–	–	–
	ECAR	–	–	–	–	–
	OCR:ECAR	–	–	–	–	–
Tormentil	TNF-α	–	–	–	–	–
(*Potentilla erecta*)	IFN-γ	–	–	–	–	–
[27B]	IL-1β	–	–	–	–	–
	IL-2	–	↓	–	–	–
	IL-4	–	–	–	–	–
	IL-6	–	–	–	–	–
	IL-8	–	–	–	–	–
	IL-10	–	↓	↓	–	–
	IL-12p70	–	–	–	–	–
	IL-13	–	–	–	–	–
	OCR	–	–	–	–	–
	ECAR	–	–	–	–	–
	OCR:ECAR	–	–	–	–	–
Cross-leaved heath	TNF-α	–	–	↑	–	↑
(*Erica tetralix*)	IFN-γ	–	–	–	–	–
[26A]	IL-1β	–	–	–	–	–
	IL-2	–	–	–	–	–
	IL-4	–	↓	–	–	–
	IL-6	–	↓	–	–	–
	IL-8	–	–	–	–	–
	IL-10	–	–	–	–	–
	IL-12p70	–	–	–	–	–
	IL-13	–	–	–	–	–
	OCR	–	–	–	–	–
	ECAR	–	–	–	–	–
	OCR:ECAR	–	–	–	–	–

Table 16. Overview of findings described in Chapters 3 & 4

The overarching theme of this thesis was to illustrate a need for further exploration into the pathophysiology of IBD and IBS, and to explore novel treatment approaches.

Despite huge advances in treating IBD over the past two decades, treatment options remain suboptimal, with evidence of a therapeutic ceiling of efficacy. Combining two drugs with different mechanisms of action is hypothesised to improve treatment outcomes however there is a paucity of data to support this approach.[1-3] Chapter 2 aimed to describe effectiveness of combination advanced therapy use for IBD in Ireland. We found it to be an effective therapeutic strategy with an acceptable safety profile in refractory IBD patients. The clinical remission rates we report are largely similar to the rates reported by Ahmed et al in meta-analysis of combination advanced therapy outcomes in IBD.[1]

To our knowledge, our study is the largest study published to date pertaining to the real-world outcomes of combination advanced therapy use in IBD. Despite our results showing moderate effectiveness associated with this therapeutic strategy, it is clear that the therapeutic ceiling of efficacy has not been breached and that further treatment strategies and modalities are required. Randomised controlled trials should be carried out to determine whether there is an optimal regimen to use for specific disease phenotypes, and whether this strategy would be more successful in a less treatment refractory group.

In Chapter 3, using a colonic explant model we reported that the inflammatory-metabolic profile of UC patients is different to that of HC and IBS patients, and that those with active UC display the greatest differences. We demonstrated reduced

oxidative phosphorylation associated with both UC in remission and active UC, suggesting altered energy metabolism in UC. Glycolysis rates positively correlated with IL-12p70 and IL-8P secretion, and also with UC disease duration. While mitochondrial dysfunction has been long described in IBD, to our knowledge this is the first report integrating real time mitochondrial respiration analysis on colonic UC explants with inflammatory protein secretion.[69] This approach allows assessment of the cross-talk that occurs between metabolism and inflammatory pathways. Our findings suggest novel therapeutic targets, including glycolysis inhibition and oxidative phosphorylation augmentation, may be beneficial in treating UC.

Microbial-derived metabolites have a key role in regulating immune cell metabolism and maintaining gut homeostasis. There is evolving evidence that the host mitochondria–gut microbiome interactions may impact the evolution of intestinal inflammation. Indeed, mice that are methylation-controlled J protein (MCJ) deficient have dysbiosis and are at increased risk of developing colitis. MCJ is a mitochondrial protein that negatively regulates ATP production. Faecal microbiota transplantation from MCJ-deficient mice to germ-free mice has been shown to increase the germ-free mice susceptibility to colitis, whereas co-housing the MCJ-deficient mice with wild-type mice demonstrated that the at-risk mice acquired a microbiome associated with reduced risk of colitis. This suggests that the microbiome and microbial metabolites may play a role in disease pathogenesis and act as a treatment target.[183] Future research should focus on the mechanism by which these metabolites control the dysregulated inflammatory and metabolic response seen in UC.

In chapter 4, we reported that three natural extracts (cuckoo flower, tormentil, and cross-leaved heath) had pleotropic effects on the colonic tissue microenvironment, including on inflammatory cytokines and metabolism. These effects differed across health and disease class. Natural compounds are known to have the potential to alter the microbiome. This can have beneficial treatment effects due to the often competitive interaction with other organisms. In light of the potential modulation of mitochondrial activity through the microbiome, further investigation is warranted into the effect that these extracts might have on the microbiome and its metabolites. Particular focus should be given on their effect on populations of known pathobionts, such as *Ruminococcus torques* which has previously been linked to IBD pathogenesis.[87,184]

Given the potentially desirable effect of cross-leaved heath on the inflammatory profile of IBS we have selected this extract for further therapeutic investigation. However, the molecular target and structure of cross-leaved heath is unknown, therefore phenotypic and target-based assays should be conducted. In addition, a combination of liquid chromatography-mass spectrometry, tandem mass spectrometry, and nuclear magnetic resonance should be considered to accurately analyse and identify the extracts properties.

The extracts we tested were dissolved in DMSO and are not currently suitable for in vivo testing; as such, the mechanism for drug delivery should be developed for cross-leaved heath. Given that these products are natural, particular focus should be paid to mechanisms that might be classed as a supplement or functional food. Such classifications streamline the delivery of drug to market. In addition, exploration

regarding the pharmacokinetics of cross-leaved heath is required to gain understanding of the bioavailability, including gut wall metabolism, hepatic clearance and renal clearance.

Our trial was small in size, with classification of IBS patients based on the subtypes of IBS. Further ex vivo testing, with an increased sample size, should incorporate different IBS subtypes such as IBS diarrhoea predominant and post-infectious IBS. TNF-α levels increased following treatment of the UC group with cross-leaved heath, therefore the potential implication of altering TNF-α secretion in an IBS cohort should be investigated. Additionally, cross-leaved heath caused a reduction in IL-4 and so further investigation should be considered in testing its efficacy for other conditions such as food allergy, skin allergy, and atopy.

While we did not select a plant extract for further therapeutic exploration in treating UC, this study highlights the difficulties encountered during drug discovery, where 90% of clinical drug development fails. Should the results from further ex vivo testing of cross-leaved heath be supportive of further exploration, in vivo safety testing will be required in tandem with further drug delivery development as stated above. Collaboration with industry is suggested to increase efficiency in bringing a drug to trial and should be considered during the next phases of this project. [185]

5.4. Conclusion

This study has identified an IBD subgroup for which novel therapy options are required. It has provided new insights into the pathogenesis of both UC and IBS, and applied a novel mechanism of experimental design to these diseases. The study demonstrated dysregulated metabolism in UC, independent of inflammation, that progresses as disease duration lengthens. Our data supports the selection of cross-leaved heath for further investigation as a treatment of IBS and potentially for non-gastrointestinal disorders such as allergic conditions. This study highlights the challenges encountered in drug discovery, particularly in the setting of incompletely defined, complex, inflammatory conditions.

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