UNIVERSITY OF BIRMINGHAM

Appraising novel host and gut microbial interactions at Inflammatory Bowel Disease onset:

Exploring the role of galectins and the microbiome

Peter Benjamin Rimmer

Thesis submitted for the degree of **Doctor of Philosophy**

Supervisors

Prof Tariq Iqbal and Prof Asif Iqbal

Institute of Cardiovascular Sciences

College of Medicine and Dentistry

University of Birmingham

i. ABSTRACT

Inflammatory Bowel Disease (IBD) is a chronic inflammatory disorder of increasing prevalence. Its aetiological basis is multifactorial, with interactions between the host immune system and gut microbiota, alongside genetic and environmental factors of key importance. Despite our increasing understanding, the consistent delivery of early diagnosis and the development of biomarkers that can predict disease course to facilitate timely personalised treatment both remain elusive.

In this thesis, I explore what is known about the gastrointestinal microbiome at IBD onset, alongside our current understanding of the immune functions of a group of proteins, beta-galactoside binding lectins, and how they relate to IBD. This is achieved via systematic review and meta-analysis relating to the microbiome, and a literature review concerning galectins.

I describe the cross-site implementation of a novel rapid-access pathway for IBD diagnosis in one of the largest NHS trusts in the UK and explore how current clinical tools can be better utilised for referral triage, resource allocation and more detailed baseline assessment. I present a comprehensive prospective analysis of how best to utilise the clinical history and tools such as Faecal Calprotectin (FCP) to optimally triage referrals. Furthermore, I introduce the first validation of the IBD Disk as a means of screening for significant psychological disturbance, whilst also identifying those at risk of adverse treatment outcomes.

Patients recruited to research from this clinical pathway provided faecal samples to allow the detailed evaluation of the treatment-naïve gut microbiome using both 16S ribosomal RNA (rRNA) sequencing and shotgun metagenomics. I have integrated this dataset with published pre-treatment IBD microbiome data to present the largest analysis of the role of microbiome at IBD onset in the literature to date. The longitudinal follow up and outcome data from our own Birmingham cohort has allowed us to build up on the findings from the pooled dataset and demonstrate novel microbial associations with disease activity, disability, and treatment outcomes.

In this same patient cohort, I have undertaken the first study of serum galectin levels in IBD patients prior to the initiation of treatment. This study has elucidated the close association of baseline galectin-9 levels to disease severity and subsequent treatment response, outperforming established clinical indices in this regard. I have, for the first time, integrated serum galectin expression with the gut microbiome at IBD onset, both in terms of overall diversity and enrichment or depletion of specific bacterial taxa. For example, serum galectin-9 levels positively correlate with the abundance of established pathobionts including *Ruminococcus gnavus*. I have developed models of this integrated data able to accurately predict the subsequent failure of conventional therapies in patients presenting with IBD.

The studies performed in this thesis contribute to the ongoing efforts to deliver earlier and personalised care to patients presenting with IBD. I aim to build upon the data presented through my ongoing research objectives, such as with targeting microbiome intervention, as I embark upon my consultant career.

ii. ACKNOWLEDGEMENTS

I am extremely grateful for the supervision and support I have received throughout this thesis. I must acknowledge the wealth of experience and know-how that I have been able to access from my supervisors, Prof Tariq Iqbal and Prof Asif Iqbal. Tariq is an exceptional physician who has been able to support me in my clinical work and provide a broad range of ideas to explore with further research. His knowledge and position as a leading mind in the study of the microbiome in IBD has helped me immensely. Asif is a brilliant scientist who has been able to guide my laboratory-based work and inspire the ideas that underpin a large part of this work.

Dr Nabil Quraishi is an inspiration. His work on the microbiome has paved the way for what I have achieved. His support and assistance with processing large sequencing datasets has been invaluable, alongside his prompt responses to my queries. I must also thank Dr Richard Horniblow and Dr Samantha Tull. Richard helped me to get to grips with and make a success of the microbial samples. Samantha has overseen the detailed cataloguing and storage of all serum samples used in this study. She has provided immense help as I have adjusted to working in a lab environment and helped to plan and execute the ELISAs undertaken in this thesis. These thanks must also extend to Prof Andrew Beggs and to his team at Genomics Birmingham, who have provided lab space for my work and provided the sequencing services. Thanks too must go to Dr Jonathan Cheesbrough and Jane Harris who have worked alongside me, to deliver our clinical pathway and research recruitment across two hospital sites, whilst Jon has also significantly helped with delivering our lab work.

Whilst I have made significant progress in my ability to undertake bioinformatic analyses, I have received support in this regard both from Dr. Alexandra Paun at F. Hoffman La Roche and Dr. Fan Zhang at the University of Sydney. I would also like to extend my gratitude to Willem Van Shaik, Ingrid Dumitriu, Amanda Rossiter, Daniel Regan-Komito, Prof. Georgina Hold, Beth Hanney, Tegan Gumsley-Read, Hannah Ireland, Melanie Love, Mandy Collins, Dr Rachel Cooney, the nursing teams in Endoscopy, the clinical research facility, and the Centre for Rare Diseases.

Final thanks must go to my long-suffering wife Imogen who has put up with my late nights and inconsistent availability whilst supporting the bringing up two children and developing a medical career of her own.

iii. STATEMENT OF ORIGINALITY

I can confirm that this thesis and the research upon which it is based that the product of my own work. Any ideas or statements from the work of others are acknowledged and cited.

I have been fortunate enough to have access to some data, described below, where I was not responsibility for the preceding laboratory work:

- Pilot galectin IBD Data from the team in Asif Iqbal's lab

Whilst I have undertaken a majority of bioinformatic work, I have had support available to take the presented analyses to a higher standard and degree of complexity. Alexandra Paun, a bioinformatician based in the Immunology, Infectious Disease and Ophthalmology team (I2O) at Roche, provided the code utilised to undertake the TOPIC analysis and Phyloseq based analyses of the Birmingham data. Dr Fan Zhang, a bioinformatician at the University of Sydney and I worked together to deliver the integrated analysis of our dataset with other publicly available sequence datasets.

iv. Table of Contents

v. List of Figures:

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Data Summary and concluding remarks

vi. List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

vii. List of Abbreviations

AIH: Autoimmune hepatitis

AUC: Area Under the Curve

AT: Advanced Therapy

BHH: Birmingham Heartlands Hospital

BMI: Body Mass Index

BRC: Biomedical Research Centre

CARD9: Caspase Recruitment Domain Family Member 9

CD: Crohn's Disease

CD40: (or any other number): Cluster of Differentiation 40

COSMIN: The Consensus Based Standards for the Selection of Health Measurement Instruments

COVID-19: Coronavirus Disease 2019

CRC: Colorectal Cancer

CRP: C-Reactive Protein

CXCL6: C-X-C Motif Chemokine Ligand 6

DADA2: Divisive Amplicon Denoising Algorithm 2

DC: Dendritic cells

DNA: Deoxyribonucleic acid

dsDNA: double stranded Deoxyribonucleic acid

ELISA: Enzyme-Linked Immunosorbent Assay

FACS: Fluorescence-activated cell sorting

FCP: Faecal Calprotectin

FDR: False detection rate

FIT: Faecal Immunochemical Test

FMT: Faecal Microbiota Transplantation

GAL: Galectin

GBP: Glycan Binding Protein

GWAS: Genome-wide association studies

H2S: Hydrogen Sulfide

HADS: Hospital Anxiety and Depression Scale

HBI: Harvey Bradshaw Index

HR: Hazard Ratio

HUMAnN: HMP Unified Metabolic Analysis Network

IBD: Inflammatory Bowel Disease
IBS: Irritable Bowel Syndrome
IQR: Interquartile range
IHC: Immunohistochemistry
IL-: Interleukin-
ILC: Innate Lymphoid Cells
ITS: Internal Transcribed Spacer
KEGG: Kyoto Encyclopaedia of Genes and Genomes
L2FC: Log2 Fold Change, the coefficient derived MaAsLin2 to express abundance changes with the sequence data
LC-MS: Liquid Chromatography-Mass Spectrometry
LPS: Lipopolysaccharide
MaAsLin2: Multivariable Association with Linear Models
MAFFT: Multiple Alignment Fast Fourier Transform
MALDI-TOF: Matrix Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry
MetaPhlAn: Metagenomic Phylogenetic Analysis
Mdiff: Estimated Mean Difference
MHD: Mental Health Disorder
mRNA: messenger Ribonucleic acid
MDP: Muramyl dipeptide
NHS: National Health Service
NIHR: National Institute for Health and Care Research
NLRP3: NLR family Pyrin domain containing 3
NMDS: Non-metric Multidimensional Scaling
NOD2: Nucleotide-binding Oligomerization Domain protein 2
OSCCAR: Ocean State Crohn’s and Colitis Area Registry
OR: Odds Ratio
OTU: Operational Taxonomic Unit
PBC: Primary biliary cholangitis
PBMC: Peripheral blood mononuclear cells
PE: Phycoerythin
PCR: Polymerase Chain Reaction
PCDAI: Paediatric Crohn’s Disease Activity Index

PICRUST2: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2

PRISM: Prospective Registry in IBD Study at MGH

PROM: Patient Reported Outcome Measure

PROTECT cohort: Predicting Response to Standardised Paediatric Colitis Therapy

PSC: Primary Sclerosing Cholangitis

QEHB: Queen Elizabeth Hospital Birmingham

QIIME2 - Quantitative Insights Into Microbial Ecology 2

qPCR – quantitative Reverse Transcription Polymerase Chain Reaction

REC: Research Ethics Committee

ROBINS-E: Risk Of Bias In Non-randomized Studies - of Exposure

rRNA: Ribosomal Ribonucleic acid

r_s : Spearman's Rho coefficient

RT-PCR: Reverse Transcription Polymerase Chain Reaction

SAA2: Serum Amyloid A2

SESCD: Simple Endoscopic Scoring System for Crohn's Disease

SGB: Species level genome bin

SR: Spearman's Rho

Tim-3: T cell immunoglobulin and mucin protein 3

TLR: Toll-Like Receptor

TNFα: Tumour Necrosis Factor Alpha

Tregs: Regulatory T Cells

UC: Ulcerative colitis

UCEIS: Ulcerative Colitis Endoscopic Index of Severity

UHB: University Hospitals Birmingham NHS Foundation Trust

UK: United Kingdom

WCC: White Cell Count

Word count (Main text excl abstract, acknowledgements, contents page, list of figures / tables, tables, figures [and legends], list of publications and appendices)

44210

CHAPTER 1: Introduction

1.1 An introduction to how this body of work came to exist

Before I explore the subject area and scientific basis for this thesis, it is necessary to provide the context and rationale for how these different components came together. This body of work starts with an opportunity, brought about by research funding and in the context of a clinical service crying out for improvement. This opening provided the springboard to transform the path to Inflammatory Bowel Disease (IBD) diagnosis within our NHS trust, integrate clinical care with research and provide novel pre-treatment research data linking the microbiome with immune pathways. The springboard I allude to owes to prior work undertaken by Prof. Asif Iqbal and Prof. Tariq Iqbal. Asif is an inflammation biologist who has long held an interest in the biology of galectins, a family of glycan binding proteins that I will discuss at length later in this introduction. This interest had originally focussed on cardiovascular and rheumatological disease. However, through a collaboration with Tariq, pilot data was generated from a heterogeneous cohort of patients with long standing IBD. This unpublished data, which I have re-analysed for this thesis, was the catalyst for a commercial collaboration and resultant research funding from F. Hoffman La Roche. This funding provided the time for me to develop and implement the clinical pathway, whilst also funding further research into galectins. Our interest in the study of the gastrointestinal microbiome as it applies to the onset and treatment of IBD owes to Tariq. The funding for this component of the thesis originated outside of our commercial collaboration, with grant support from Guts UK and latterly allocated funds from the National Institute for Health and Care Research (NIHR), via the Birmingham Biomedical Research Centre 2023 grant. I hope to demonstrate via this thesis the positive impact I have had through capitalising on this opportunity.

1.2 Key concepts in Inflammatory Bowel Disease

IBD refers to a spectrum of conditions, classically divided into Ulcerative Colitis (UC) and Crohn's Disease (CD). It is an immune mediated disease, whereby inflammation of the intestinal mucosa results in a significant burden of gastrointestinal symptoms. Whilst presenting symptoms are diverse, the presence of diarrhoea, particularly in combination with the passage of blood is classical. Symptoms are not confined to the digestive tract, with extra-intestinal manifestations being common, fatigue and joint pain frequently reported (Perler et al., 2019). In the conventional form, the hallmark of UC is continuous symmetrical inflammation, usually starting in the distal large intestine, confined to the colonic mucosa. Histologically, the active phase of the disease is characterised by cryptitis and crypt abscesses (DeRoche et al., 2014). CD can affect any part of the digestive tract. It is characterised by the potential for transmural intestinal inflammation and consequent complications including the formation of inflammatory and fibrotic strictures and fistulae. Histologically, non-caseating granulomas may be present as well as features such as fissuring ulceration and transmural lymphoid aggregates (Tanaka et al., 1990). Though the separation of these conditions into their two subtypes remains the standard clinical practice, it is recognised that the margins of the two conditions are blurred, with for example, CD isolated to the colon sharing more features with UC patients than those with CD isolated to the ileum (Soucy et al., 2011). Furthermore, IBD associated with Primary Sclerosing Cholangitis (PSC) behaves in a very different manner to traditional UC and is accordingly likely to have its own separate aetiology (Selin et al., 2021).

1.2.1 Exploring the complex relationships that underpin IBD.

Within urban societies, the incidence and prevalence of IBD is increasing rapidly. Research from within the United Kingdom (UK), currently unpublished in peer reviewed form, estimates that 0.81% of adults are living with IBD (Crohn's & Colitis UK, 2022). It is further suggested that this is like to increase to 1.1% by 2025 (King et al., 2020). Societies undergoing industrialisation show rapidly increasing incidences of IBD, whilst emigration to industrialised society is an independent risk factor for developing IBD (Alatab et al., 2020; Acosta et al., 2011). These changes have led to interest in environmental factors and their role in IBD onset. In truth, the picture is more complex, with multiple factors interacting together to underpin IBD onset. There is a growing burden of evidence that IBD represents the combined efforts of dysregulated host immune responses, driven by a genetic pre-disposition and initiated by specific triggers within the environment (Selin et al., 2021).

These triggers may be those in early life, such as antibiotic exposure and method of feeding (Agrawal et al., 2021). They may also be more incremental, repeated exposures for example from diets high in refined sugars and emulsifiers or pollutants more prevalent in urban environments (Ananthakrishnan et al., 2018). These events and exposures have been shown to impact on the gut microbiome. More abrupt events are also relevant, for example through the impact of antibiotics, or episodes of infective gastroenteritis , both of which can have a profound influence of the microbiome and have been associated with an increased risk of IBD development (Willing et al., 2001; Rodriguez et al., 2006). Whatever the event, disruption of the 'healthy' microbiome is characterised by a reduction in diversity – the overall number of bacterial species present within the community. Overall, there is a loss of usually

abundant beneficial species, such as short chain fatty acid producers, and an increase in pathobionts that have the potential to behave in a damaging manner. The presence of these changes has traditionally been termed ‘dysbiosis’. Whilst this can now be viewed as a major oversimplification of a complex process, it has been considered a hallmark of IBD in the published literature (Mullish et al., 2021).

Microbiome disruption alone is not enough to cause IBD. First, it is seen in other conditions, such as Irritable Bowel Syndrome (IBS), where colonic inflammation is absent (Wang L et al., 2020). Furthermore, ‘dysbiosis’ in IBD has been shown to represent a dynamic process when mapped longitudinally, linking to disease flares (Halfvarson et al., 2017). It is plausible that some of these changes could be ’field changes’ resulting from an inflammatory environment. Intestinal homeostasis is reliant on several immune mechanisms in addition to preserved intestinal barrier function and mucous layer. Dysregulation of these processes can increase opportunities for pathogenic organisms and increase their interactions with immune cells. Genome-wide association studies (GWAS) have identified multiple risk loci specific to IBD but have also emphasised that there is not one single gene to target – with more than 240 risk loci associated with IBD at present after an additional 20 were recently discovered (De Lange et al., 2017; Cordero et al., 2023). Some genes, associated with very early onset IBD, may exhibit mendelian inheritance, but most do not, with heterogenous adult cohorts having a broad range of genes and pathways implicated (Graham and Xavier, 2020). ***Figure 1 – 1*** concentrates on the functions of several IBD risk genes but also focusses on some of the key immune pathways that underpin the inflammatory processes seen in IBD. Both the innate and the adaptive immune system are implicated in IBD (Kmiec et al., 2017).

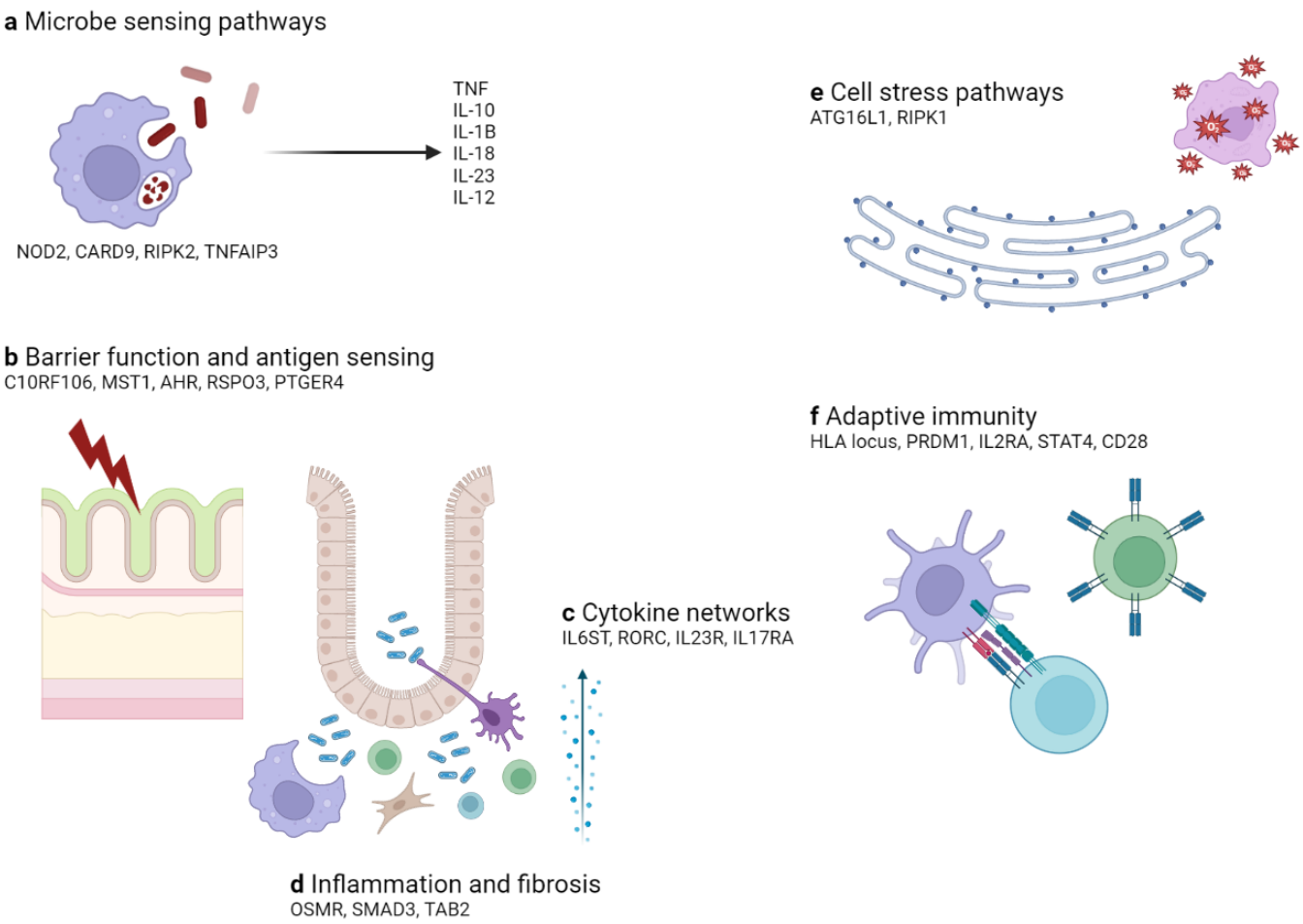


Figure 1 - 1: Selected IBD risk genes and impacted immune pathways (adapted from Graham and Xavier, 2020).

This figure, created in Biorender, presents a selected but not exhaustive list of IBD risk genes as described in the above publication.

A. As shown in **(a),** genes associated with microbe sensing pathways are perhaps the most well-known, particularly Nucleotide-binding oligomerisation domain-containing protein 2 (NOD2), Caspase recruitment domain-containing protein 9 (CARD9), Receptor-interacting serine/threonine-protein kinase 2 (RIPK2) and Tumour necrosis factor, alpha-induced protein 3 (TNFAIP3).
B. Genes influencing the maintenance of epithelial barrier function, antigen sensing **(b)** and the ability of junctional complexes to remodel after injury are also prominent and include C1orf106, Macrophage Stimulating 1 (MST1), Aryl hydrocarbon receptor (AHR), R-spondin 3 (RSO3) and Prostaglandin E Receptor 4 (PTGER4).
C. Genes associated with Inflammatory pathways modulating cytokine release **(c)** including Interleukin-6 signal transducer (IL6ST), RAR-related orphan receptor C (RORC), Interleukin-23 receptor (IL23R) and Interleukin-17 A receptor (IL17RA) have been linked to IBD.
D. Genes associated with inflammation and fibrosis **(d)** are similarly linked, including Oncostatin M receptor (OSMR), Mothers against decapentaplegic homolog 3 (SMAD3) and *TAB2*.
E. Genes that increase vulnerability in integrated stress response pathways **(e)** have been implicated in the onset of IBD, including Autophagy related 16 like 1 (ATG16L1) and Receptor-interacting serine/threonine-protein kinase 1 (RIPK1).
F. Finally, genes linked to adaptive immune responses **(f)**, including Human Leucocyte Antigen (HLA) locus, PR domain zinc finger protein 1 (PRDM1), Interleukin-2 A reception (IL2RA), Signal transducer and activator of transcription 4 (STAT4) and Cluster of Differentiation (CD) 28 have been associated with an increased risk of IBD.

Innate responses are directed against pathogens with microbe sensing occurring via receptors including NOD2 and Toll-like receptors (TLRs). NOD2 functions by sensing cytosolic muramyl dipeptide (MDP), a peptidoglycan derived from bacterial cell walls. When MDP is engaged by NOD2, activation of Nuclear factor-κB ions occurs, in turn promoting IL-1B secretion (Martinon et al., 2004). Flagellin and Lipopolysaccharide (LPS) may also be sensed. Dendritic cells (DCs) in health produce retinoic acid during the imprinting of naïve T cells in the intestinal mucosa, inducing molecules such as integrin α4β7 and aiding the generation of Foxp3+ regulatory T cells (Selin et al., 2020). This process is seen to break down in IBD patients, with reduced numbers of retinoic DCs seen (Magnusson et al., 2016). Furthermore, DCs express higher levels of TLRs in IBD, with consequent increases in microbial sensing supported by increased activation seen via increases in CD40, as well as IL-6 and IL-12 expression (Hart et al., 2005). Innate lymphoid cells (ILCs) are also thought to serve important functions in interactions with both intracellular and extracellular microbes, amongst other pathogens. ILCs mirror the functions seen in T cells and in IBD IL-23 drive cytokine and granulocyte-macrophage colony stimulating factor secretion. IL-17 and interferon- γ producing ILCs are selectively increased in the inflamed mucosa of patients with CD (Geremia et al., 2011).

Adaptive immune pathways play a key role in the establishment of chronic inflammation in IBD patients. Regulatory T cells (Tregs) are a key controller of intestinal immune homeostasis and accordingly, a large degree of focus has been placed upon their role in IBD pathogenesis (Selin et al., 2021). Several papers have demonstrated increases in Tregs in inflamed mucosa, increasing in number with increasing severity. Furthermore, these cells have been shown to have a reduced

ability to suppress T cell proliferation (Ueno et al., 2013) and increased rate of apoptosis (Veltkamp et al., 2011). CD4+ T helper (Th1, Th2 and Th17) lymphocytes have also been associated with the pathogenesis of IBD. All show interactions with pathogens and carry a more proinflammatory phenotype. Th1 cells produce interferon- γ and TNF, a key facilitator of intestinal barrier dysregulation in IBD (Imam et al., 2018; Nava et al., 2010). Th17 cells are IL-17 producing cells that play a key role in the regulation of commensal bacterial populations at interface sites such as the gut mucosa. However, dysregulated responses are seen in IBD with increased IL-17 triggering further secretion of associated cytokines and chemokines that are elevated in the mucosa of IBD patients (Kmeic et al., 2017). A recent study undertaking single cell sequencing of mucosal biopsies from IBD patients at disease onset has shown increases in CD4+ T cells within the colonic mucosa in CD, though interestingly not the ileum. Furthermore, the highest abundance was seen in the non-inflamed biopsies from the patients with CD (Kong et al., 2023).

1.3 Shining a spotlight on the gut microbiome

The study of the microbiome is a well-established subject across multiple scientific domains. The origins of the terms ‘microbiota’ and ‘microbiome’ are often stated to extend back to the work of Lederberg and McCray (2001). This is a source of quite significant debate, with others pointing to their established use within the field of microbiology as far back as the 1960s (Prescott, 2017). In the context of current paradigms, ‘microbiota’ is used to refer to the total accumulation of up to 100 trillion microbial cells which can exist within a given environment, whilst ‘microbiome’ refers to the total ecological habitat in a given community, inclusive of their genomes

(Mullish et al., 2021). In the context of our work, the microbiome found within the human gut is the area of interest, but the same terminology can be applied to the microbial communities found it any ecological habitat. The human gut microbiome is known to contain more than 100 trillion microorganisms, with an estimated 10^{11} - 10^{12} per millimetre (Rinninella et al., 2019). Present organisms are known to encompass bacteria, viruses and fungi amongst other prokaryotes. In the context of health, these microbial communities are known to interact collaboratively with the host. However, perturbations in these communities are seen in association with multiple disease processes.

1.3.1 What is a healthy gut microbiome and how do we define it?

Defining '*healthy*' in the context of the gastrointestinal microbiome is complex. First we must consider whether *healthy* is defined by the absence of disease, or more stringently, by the absence of gastrointestinal complaints outside of set diagnostic criteria (Van Hul et al, 2024). Once this has been agreed upon, it is necessary to consider which component of the microbiome is to be focussed upon. This should not only encompass composition, but also the functional capacity of the microbiota, for example their production of metabolites such as short chain fatty acids. Thereafter, variations driven by other external factors such as geography must be factored in and work continues to identify core microbial populations that define health across different populations (Falony et al., 2016).

Arumugam et al. (2011) describe the dominant human gut microbial flora as predominantly consisting of the phyla *Firmicutes* and *Bacteroidetes,* validating this with samples across multiple continents. These phyla are seen to constitute more than 90% of the gut microbiome. *Clostridia* make up 95% of the firmicutes phyla, whilst Bacteroidetes are largely *Bacteroides* and *Prevotella*. Other contributing phyla include *Actinobacteria, Proteobacteria, Fusobacteria* and *Verrucomicrobia* . The gut microbiome in health has been extensively characterised in North American participants. The Human Microbiome Project was able to characterise the microbiome of 242 individuals at multiple body sites and demonstrated high levels of microbial diversity as a key feature of health, particularly within the gastrointestinal tract (Huttenhower et al., 2012). Ongoing works aim to provide a more global perspective but remain dominated by samples from European and North American participants (Abdill et al., 2025). Many of the high abundance *Firmicutes* seen in health are associated with the production of short chain fatty acids such as butyrate through carbohydrate metabolism, which in turn is used by colonocytes to generate energy, maintain mucosal barrier integrity and inhibit inflammatory pathways (Singh et al., 2023).

The acquisition of the gut microbiome is a dynamic process driven by early life events. The neonatal microbiome is characterised by relatively low diversity, dominated by *Bifidobacterium* (particularly *Bifidobacterium breve*) and influenced by exposures such as mode of delivery and breast feeding (Püngel et al., 2020). The first 1000 days of life are viewed as the most important, with the microbiome thereafter reported to resemble that seen in adulthood (Wopereis et al., 2014). Whilst

this concept is often cited, other authors have presented evidence of continued evolution in the composition of the microbiome through childhood into teenage years. This has been shown in healthy individuals across both microbiome structure and function (Hollister et al., 2015; Radjabzadeh et al., 2020), with evidence of difference even extending to adolescence (Agans et al., 2011).

Other important environmental factors and exposures that can impact upon the microbiome include exposure to medications such as antibiotics, exposure to environmental pollutants and diet, with ultra-processed foods high in emulsifiers and sweeteners known to have negative impacts on the microbiome (Miclotte et al., 2020; Suez et al., 2022). It has been shown that humans living in industrialised society harbour the lowest gut bacterial diversity of any primate where sequencing has been undertaken. In human population studies, the greatest loss of microbial diversity is seen when people move from agricultural to urbanised lifestyles (Moeller, 2017).

The factors influencing gut microbiome composition across a human life span, including the first 1000 days, is depicted in ***Figure 1 – 2.***

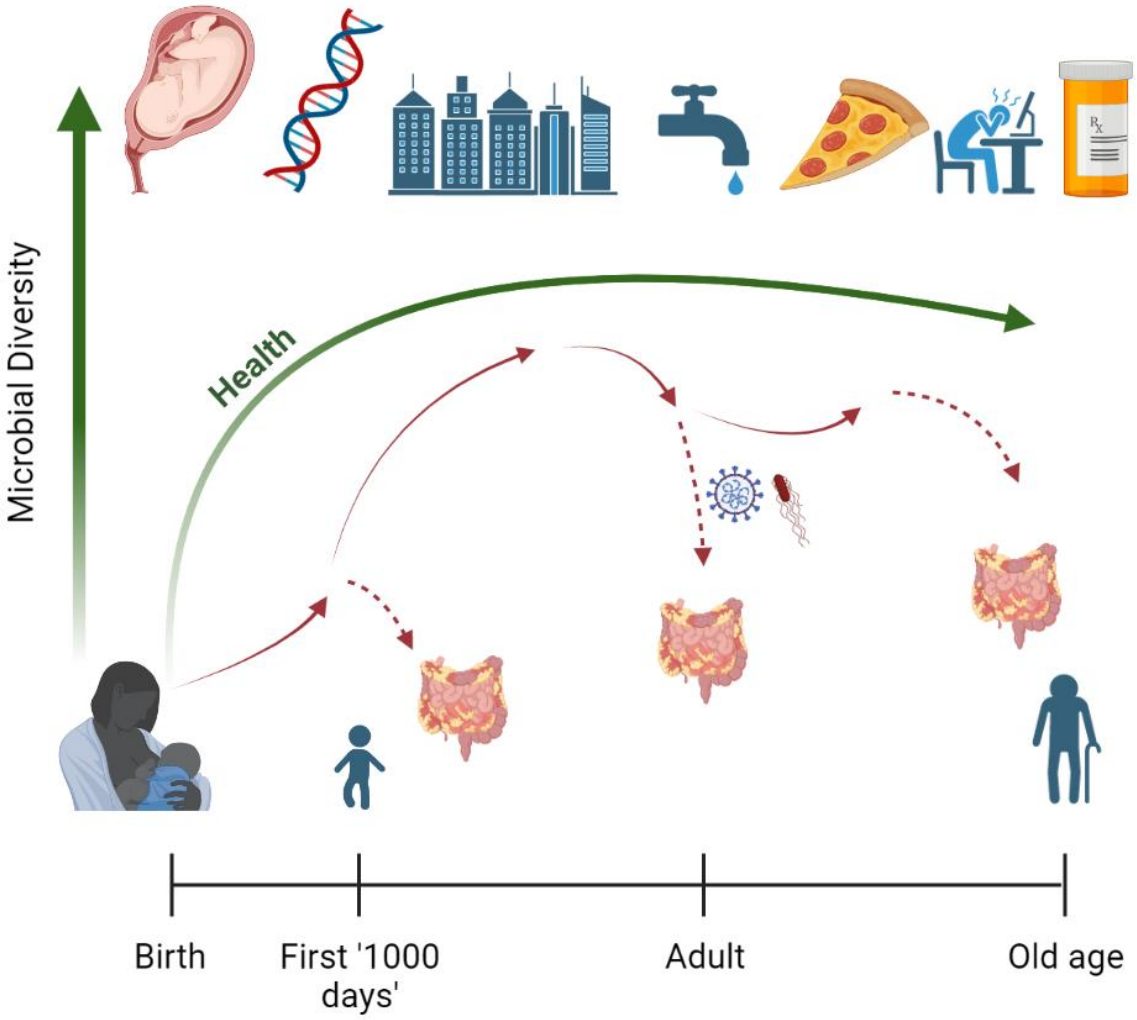


Figure 1 - 2: Microbiome acquisition and determinants of stability (adapted from Kostic et al., 2014)

The early life microbiome transitions to a more adult state within the first 1000 days. Genetics, mode of birth, breast feeding, and antibiotic exposure are among key determinants than can increase future risk of IBD. Ongoing factors such as geography, hygiene, western diet, stress, medication use and acute infective episodes such as gastroenteritis are known to exert significant effects on the microbiome, which can be associated with IBD onset. Diversity is known to decline in later life regardless of external factors. This figure was created in Biorender.

When studying the microbiome, there are some core parameters that must be considered. First is the overall diversity of the microbiome. Second is the nature of the component parts that make up a given community. There are two forms of diversity that are of interest, both coined by Whittaker (1960, 1972). Alpha diversity refers to the overall structure of an ecological community in terms its number of different taxonomic groups (also referred to as richness), the abundances of these groups (also referred to as evenness) or a combination of both. The other form is beta diversity. This term, subject to much debate in recent years, refers to a quantification of the differences in operational taxonomic composition between two ecosystems. In the setting of microbiome studies, this would refer to a direct assessment of difference between two given samples.

There are multiple different methods and measures available to calculate a value to represent these concepts. Methodological variation and at times, omission of core indices, is a recurring theme within the literature in this field. Without an understanding of the subtleties associated with these measures, interpretation can be challenging.

1.3.2 Current approaches to sequencing the gut microbiome

Over the course of the past 20 to 30 years, the field has progressed rapidly and enabled huge developments of our understanding of both the constituents of the microbiome, but also their importance in the regulation of health and disease. The field has moved away from traditional culture-based approaches of trying to grow

given organisms. These methods tended to focus on a single pathogenic organism in the context of acute illnesses and were biased by the inability to culture multiple different organisms, leaving us unable to determine community structures (Weinstock, 2012). With the development of high throughput sequencing technologies this has changed, with multiple techniques available to determine both the community structure of the microbiome and its functional capacity (Quince et al., 2017). Next generation sequencing technologies allow for the extraction and analysis of vast quantities of taxonomic and genomic data. Current techniques frequently utilised within the medical literature are metataxonomics and metagenomics.

Metataxonomics involves sequencing the 16S rRNA gene following amplification by polymerase chain reaction (PCR). The 16S rRNA gene is present in the genome of all prokaryotes. Conservation of the gene sequence relationships of 16S rRNA was first noticed over 50 years ago (Dubnau et al., 1965). It is now understood to consist of highly conserved regions interspersed with hypervariable regions, which have differing sequences and total length between different bacteria. Amplification of these regions by PCR (for example, the V4 region is commonly chosen when using an Illumina sequencing platform [San Diego]) allows the identification of different bacterial genes. Definitions of given bacterial species tend to be made on the presence of at least 97% of the identity of a known operational taxonomic unit (OTU) (Weinstock, 2012). Using unique indexed primers, it is possible to sequence multiple samples at once and separate individual results using these unique identifiers. Such approaches allow reliable determination of microbiome community structure to a genus level at relatively low-cost. However, datasets are subject to different biases

depending upon PCR and sequencing technique used. It is not possible to reliably identify bacteria at a depth beyond genus and some bacteria with different copy numbers are sequenced less well, with artificial influences on overall abundance (Mullish et al., 2021).

Shotgun Metagenomics refers to the untargeted sequencing of all the microbial genomes that are present in a sample. Microbial DNA is fragmented and all DNA fragments within the sample are sequenced. This approach can determine taxonomic composition, including low abundance taxa if adequate read depths are utilised, down to species and strain level. In doing so, supplementary data such as that relating to the functional activity of a given microbial community, can be derived (Quince et al., 2017). Host genomic data will be included with sequencing outputs and can be targeted for evaluation or filtered. Though knowledge in this area is continually growing, gaps in reference microbial databases may make some sequencing data challenging to map and interpret.

1.3.3 Core indices underpinning microbiome analysis

The best approach to the measurement of both alpha and beta diversity is hotly debated, with a wide variety of indices already developed. Regarding alpha diversity, common indices include, but are not limited to:

- Species richness: The overall number of species present within a sample. This approach is flawed with too strong an association with sample size, whereby smaller cohorts, with fewer samples, will present fewer species by probability

alone, regardless of whether a biological difference in community structure is present. (Roswell et al., 2021).

- Chao1 index: A nonparametric measure of species richness developed be Anne Chao (1984). It places greater emphasis on low abundance species, enhancing its utility in datasets with low abundance factors (Kim et al., 2017).
- Simpson index: This measure, originally described in 1949, includes both species richness and relative abundance (Simpson, 1949).
- Shannon Entropy: This index is one of the commonest indices within medical microbiome literature. It follows a similar premise to the Simpson index. However, the units change on an entirely different scale to Simpson with differences in species abundance, making interpretation difficult (Shannon and Weaver, 1963 and Roswell et al., 2021). To overcome this discordance, an 'inverse Simpson', which correlates positively with Shannon, is used by some authors.
- Faith's phylogenetic diversity: This does not consider abundance. It relates specifically to the breadth of the phylogenetic tree present within an observed population and is expressed as the total branch length covered by a community (Faith, 1992).

Concerning beta diversity, common indices include, but again are not limited to:

- Bray-Curtis Dissimilarity: Measures the dissimilarity between two different sites, or from a microbiome perspective, the number of shared taxa between two groups (Bray and Curtis, 1957).
- UniFrac distance (weighted and unweighted): This quantifies the degree of separation between communities of taxa within specified groups or samples. The

distance referred to relates to the observed branch length within in a phylogenetic tree that leads to descendants from either one group or other, not both (Lozupone and Knight, 2005).

- Jaccard similarity index: A ratio, expressed as one minus the ratio of the intersection of present / absent OTUs between two samples (Jaccard, 1912).

When examining datasets at varying taxonomic levels, statistical methodology is also of vital importance. Whilst there are a multitude of bioinformatic pipelines available, it is essential that any conclusions drawing about differences in relative abundance are accompanied by a false discovery rate (FDR) or q value. In large studies utilising high throughput sequencing technologies, multiple comparisons of different parameters and taxa are undertaken across multiple samples. This poses an issue for a traditional p value. A p of 0.05 equates to 5% false positive rate across all tests. In a dataset involving running 1000 tests across participants and community components a p value may result in 50 false positives. As the dataset scales up, so too does the size of this issue in interpretation. A FDR corrected q value attempts to overcome this. It presents the ratio of false positives specifically to the number of overall significant results (Noble, 2009). As such, in its simplest terms, a q-value of 0.05 states that only 5% of the tests that were found to be statistically significant by the p-value will be false positives. A common package utilised in microbiome studies, MaAsLin2, calculates multivariable associations between microbial meta'omic features utilising general linear models, the correlation coefficients of which can be used to confer Log2 fold change, referred to subsequently as L2FC (Mallick et al., 2021). Prior high impact papers utilising this pipeline have presented FDR corrected q values of <0.05 as 'strong associations', whilst <0.2 is often used for 'weak associations' (Schirmer et al., 2018).

1.4 A systematic review and meta-analysis of the pre-treatment gut microbiome at IBD inception

To capture the cleanest view of the IBD associated microbiome, prior to the development of engrained inflammatory pathways and multiple inseparable treatment effects, it is necessary to study it as close to diagnosis as possible, prior to the initiation of pharmacologic therapy (Pu et al., 2022). Whilst it is acknowledged that the interaction between host immune factors and gut microbiota is a key component in the pathogenesis of IBD, the extent to which disturbances in the microbial community are a cause of disease or a reaction to an inflamed mucosal environment remains controversial (Fritsch and Abreu, 2019). It is known, for example, that functional disease processes such as irritable bowel syndrome (IBS), are also associated with significant dysbiosis (Wang et al., 2020). In this context, changes in microbial community structure alone can't be considered the sole cause of IBD.

By comparing the microbial communities of defined cohorts with pre-treatment IBD against control groups, including 'healthy' individuals and symptomatic individuals without IBD, it may be possible to identify key differences. This has been a focus in the literature for some time. The earliest studies in this field focused on attempts to identify a single causative organism. As far back as the 1960s, Gorbach et al. (1968) referred to the unfruitful attempts to identify a single organic cause by colleagues in the field (Weinstein, 1961; Kirsner and Palmer, 1954). As early as this, the consideration of microbial community imbalance was put forwards as a putative cause. Gorbach and colleagues characterised the faecal microbiota of 17 patients

with ulcerative colitis and 8 with regional enteritis prior to treatment using culture-based techniques, identifying increases in the number of coliforms in patients with severe colitis and regional enteritis. Whilst these studies, and the work of other pioneers such as Schumacher et al. (1993 and 1995) and Hartley et al. (1992 and 1993) provide fascinating insight into the growth of the field, we now understand that culture bias limits our understanding of complex microbial communities. The advent of molecular (DNA-based) methodologies, particularly next-generation sequencing has led to a wealth of novel research in this sphere (Margulies et al., 2005). The first studies to utilise high throughput technologies in IBD began to appear in the literature around 2006 (Gophna et al., 2006). The first study to present next-generation sequencing data from a pre-treatment cohort was seen in 2012 (Kellermayer et al., 2012). The expansion of the body of literature in this space has been rapid, but as both sequencing and bioinformatic technologies have moved at pace, methodological standardisation is non-existent (Mirzayi et al., 2021). This key limitation, alongside a paucity of studies in treatment naïve cohorts, renders reliable conclusions regarding microbial disruption elusive.

With this in mind, we have undertaken a systematic review and meta-analysis of published alpha diversity data and supplemented this with thematic analysis of descriptions of differential abundance. Alongside this, we have quantified the degrees of methodological variation that underpin the conclusions being drawn. Later in this thesis I will present an integration of our own microbiome data with the publicly available raw sequence datasets that were identified and obtained during this review process.

1.4.1 Methods

1.4.1.1 Search strategy and study identification

At conceptualisation, the review was registered on PROSPERO (registration ID: CRD42022371173, 28 October 2022). The study was carried out in line with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Page et al., 2021). The search strategy was developed and piloted with the support of an information specialist and is available in **Appendix A**. Both free-text and MeSH terms were utilised with searches conducted on Medline and Embase between inception and December 2022. To reduce publication bias, ongoing studies were sought from controlled-trials.com, clinicaltrials.gov and supplementary materials published online. Abstract only publications were eligible if sufficient information to judge inclusion was provided. Hand searching was undertaken of references in articles reviewed and relevant grey literature. Conference proceedings over the preceding 12 months were also screened.

All experimental and non-experimental study designs involving both adults and children were considered. Studies undertaking analysis of the gastrointestinal microbiome in a defined newly diagnosed, treatment-naïve cohort of inflammatory bowel disease patients were eligible for initial inclusion. The treatment-naïve state was defined by sampling prior to the initiation of conventional pharmacological therapy. Suitable sample types for microbial analysis included faecal samples, mucosal biopsies, gastrointestinal washings and oral samples (including saliva, plaque and mucosa). Both ‘healthy’ asymptomatic control populations and symptomatic ‘non-IBD’ populations were utilised as comparators. In cases where there was insufficient data to judge inclusion, primary authors were contacted for

further information. Where there was disagreement, this was resolved by discussion and consensus.

1.4.1.2 Outcome assessment

Primary outcomes were to establish and quantify perturbations in the gastrointestinal microbiome at IBD diagnosis and across disease phenotypes. This included:

- population-based changes in alpha diversity
- specific microbial taxa associations with specific diagnoses and disease severity.

1.4.1.3 Data extraction

All extractions were done independently in duplicate by two researchers into a Microsoft Excel Spreadsheet. For each study, extracted data included: country of origin, age, cohort, diagnosis and control type, sample type, sample storage, DNA extraction method, sequencing type, sequence domain, read depth, bioinformatic tools utilised, correction for multiple testing, alpha diversity (and indices presented), beta diversity (and indices presented), phylum to family abundance data, genus to strain abundance data, data relating to microbial function, microbial relationship to disease activity and treatment outcome and any other ‘omic’ data presented.

1.4.1.4 Quality assessment

The Risk Of Bias In Non-randomized Studies - of Exposure (ROBINS-E) tool examined the strength of evidence about the presence of, and/or nature of, the potential effect of exposure (IBD) on outcomes. A priori, key potential confounders were agreed for use, including age, gender, BMI, baseline diet and prior antibiotic

use. The outcomes for signalling questions were reported and the overall risk of bias judgement.

1.4.1.5 Data synthesis

Two forms of data synthesis were undertaken. First, meta-analysis of published alpha diversity data was undertaken only on data derived from next generation sequencing, given traditional PCR based values were not considered comparable. All diversity measures including measures of central tendency and spread (e.g. mean and standard deviation) were collected. Different diversity measures were not combined in analysis. Exact values, and associated measures of spread were retrieved from each publication. Where plots were presented without stated values, these were inferred using a web-plot digitizer (Rohatgi, 2021). This allowed the inference of approximate values using presented scales and marked points within plots. Where only median and IQR were presented, the mean was taken to be the same as the mean and the IQR divided by 1.35 to estimate standard deviation, as described in the Cochrane handbook (Higgins et al., 2019). We performed meta-analyses of defined alpha diversity indices only where 2 or more studies were available using the same diversity measure and when patient groups and outcomes were sufficiently similar. We expressed the diversity measure as mean difference (MD) with 95% CIs. Inconsistency was quantified and represented by the I^2 statistic. For interpretation, the Cochrane handbook was employed, a result of less than 40% was deemed as not important, 40-75% may represent heterogeneity and >75% indicating considerable heterogeneity. Statistical analyses were performed using Review Manager, version 5.4 (The Cochrane Collaboration, 2020).

The second approach was thematic analysis of all available full text manuscripts. All identified texts were uploaded to the MAXQDA software package (VERBI software, 2021). Overall word frequencies were calculated across all documents. These were subsequently manually filtered to leave only words relating to microbial taxa at genus level and below. The top 25 genera were then analysed across all papers to draw out themes regarding differential abundance, with attention given to species level differences relative to controls, alongside associations with disease activity, treatment outcome and microbial function within these genera. Genera were only included if mentioned on more than 10 occasions across 10 or more papers.

1.4.2 Results

1.4.2.1 Search screening and inclusion

The search strategy identified 12264 studies, with 8 further studies identified from other sources. 93 studies were identified that presented data from defined cohorts of pre-treatment IBD patients. 61 of these were full text publications, with 32 conference abstracts. All full texts were included in the thematic analysis. However, after applying stricter criteria and excluding all studies not presenting data derived from next generation sequencing, it was possible to include only 19 studies in the synthesis of alpha diversity indices. The study PRISMA is shown in ***Figure 1-3***.

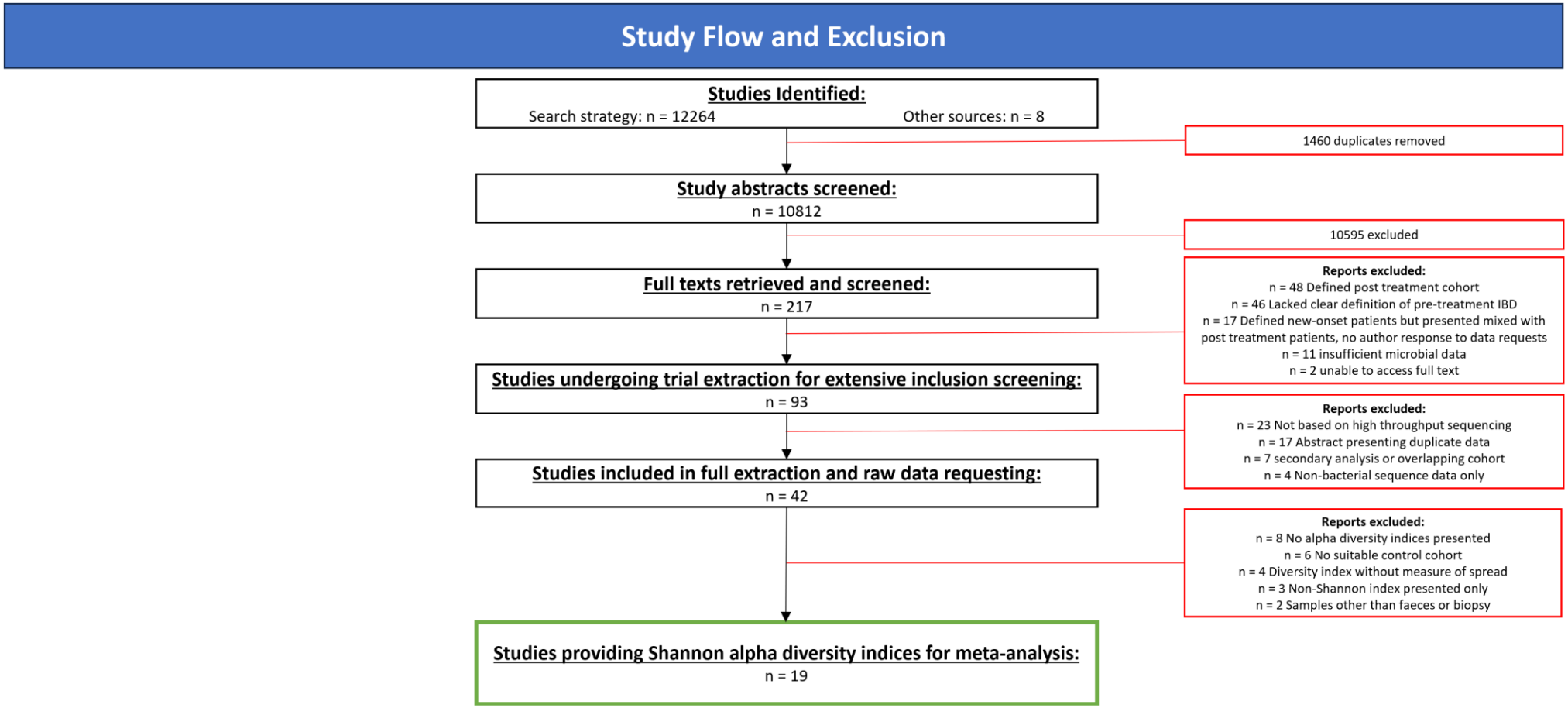


Figure 1 - 3: Study identification and filtering prior to data extraction

A total of 12264 abstracts were identified using the chosen search strategy relating to the microbiome and IBD, in addition to 8 studies from other sources. 1460 duplicates were removed and 10812 abstracts screened. After review, 217 full texts were screened with 61 full texts and 32 abstracts contributing to some component of the work. Ultimately, only 19 studies contributed to the alpha diversity synthesis, as shown in Table 1-1. All full texts were included in the thematic analysis, as shown in Appendix A.

1.4.2.2 A meta-analysis of alpha diversity at IBD onset: Results

21 studies presented alpha diversity indices alongside a measure of spread from IBD patients and controls. Of these, 19 (86%) presented the Shannon index, which was then utilised in this synthesis. Study summary characteristics are in ***Table 1-1***. IBD was stratified by subtype, whilst controls were stratified the presence of symptoms ('Healthy' controls without symptoms [HC], 'symptomatic' with symptoms but IBD excluded [SC]). 9 studies presented data from CD, 4 from UC and 6 from both. All studies utilising mucosal biopsies compared IBD with SCs, whilst those using faeces samples compared with HCs. Samples originated from four continents (12 countries). Methodological variation was marked. 12 different DNA extraction kits were utilised, sequencing performed using 4 different platforms and targeting 7 different 16S rRNA hypervariable regions. Bioinformatic approaches varied and were inconsistently reported, preventing a more systematic approach to description. The most reported pipelines were QIIME (11 studies) followed by Mothur (4 studies). 8 studies were mapped using the Greengenes database, whilst 6 used SILVA. Sequence depths varied significantly. Important factors such as sample acquisition (fresh frozen vs stored with a buffer) and storage (duration of time frozen and temperature, number of freeze-thaws) method were seldom reported. In total, the samples from the included studies encompassed 553 IBD patients (CD 411, UC 138, IBD-U 4) and 515 controls (Symptomatic 267, 'healthy' 248).

Summary ROBINS-E judgments for signalling are included within ***Table 1-1***. The full tool use for assessments, with individual ratings, can be found within **Appendix A.**

Table 1 - 1: Individual details and methodological approaches for studies included in alpha diversity synthesis.

AUTHOR	YEAR	COUNTRY	DIAGNOSIS # SUBJECTS WITH DATA	AGE GROUP	AGE GROUP Overall in study Mean (SD)	 Median (IQR)	SAMPLE TYPE IN ANALYSIS	EXTRACTION KIT	SEQUENCING METHOD	PLATFORM	DOMAIN	DEPTH MEAN UNLESS STATED	DIVERSITY INDICES	RISK OF BIAS ROBINS E
Kellermayer et al.	2012	USA	CD 15 SC 26	Paediatric	CD 13.4 (ND) SC 12.7 (ND)		Mucosal biopsy	Qiagen QIAamp mini kit	Amplicon	Roche 454	V1-3	5200	Shannon Chao1	Low
Hansen et al.	2012	Scotland	CD 11 UC 11 SC 12	Paediatric		CD 14.2 (ND) UC 13 (ND) SC 11.4 (ND)	Mucosal biopsy	Qiagen QIAamp mini kit	Amplicon	Roche 454	V3-4	21691	Shannon Simpson Chao1 Phylogenetic Diversity Observed species	Low
Perez-Brocal et al.	2015	Spain	CD 8 SC 20	Adult	CD 45.4 (18.1) SC 33.5 (14.4)		Faeces	Qiagen QIAamp DNA stool mini kit	Amplicon	Roche 454	V1-3	10043	Shannon Chao1	Some concerns
Mottawea et al.	2016	Canada	CD 20 UC 8 SC 9	Paediatric		CD 14 (3.3) UC 15 (4.5) SC 14 (6)	Mucosal biopsy	FastDNA Spin Kit	Amplicon	Illumina HiSeq	V6	200000	Shannon Chao1	Low
Assa et al.	2016	Canada	CD 10 SC 15	Paediatric		CD 14 (4.8) SC 14 (2)	Mucosal biopsy	FastDNA Spin Kit	Amplicon	Illumina HiSeq	V6	292215	Shannon Chao1	Low
Shah et al.	2016	USA	UC 10 SC 13	Paediatric	UC 12.9 (3.7) SC 13.9 (1.8)		Mucosal biopsy	Qiagen 'buffers'	Amplicon	Illumina MiSeq	V4-6	2350 rarefied	Shannon Observed species	Low
Shaw et al.	2016	USA	CD 11 UC 3 HC 4	Paediatric	ND	ND	Faeces	Not stated	Amplicon	Illumina MiSeq	V4	6600 median	Shannon	High
Fossum Moen et al.	2018	Norway	UC 44 SC 35	Adult		UC 35 (ND) SC 35 (ND)	Mucosal biopsy	Qiagen AllPrep mini kit	Amplicon	Illumina MiSeq	V4	At least 3000	Shannon	Low
Kowalska-Duplaga et al.	2019	Poland	CD 63 HC 18	Paediatric	*Given in months:* CD 151.4 (42.4) HC 138.3 (35.2)		Faeces	Genomic Mini AX stool spin kit	Amplicon	Illumina MiSeq	V3-4	23662	Shannon Observed OTUs Faith's phylogenetic diversity Pielou's evenness	Low
Kansal et al.	2019	Australia	CD 88 SC 66	Paediatric		CD 12 (ND) SC 12.3 (ND)	Mucosal biopsy	Qiagen AllPrep mini kit	Amplicon	Illumina MiSeq	V2	9188	Shannon Chao1 Observed taxa Gstat	Low

Tang et al.	2021	China	CD 25 HC 12	Paediatric	'Responder' 12.3 (0.4) 'Nonresponder' 9.3 (1.6) SC ND		Faeces	Qiagen QIAamp stool mini kit	Amplicon	Illumina MiSeq	V3-4	ND	Shannon	Low
Wang X et al.	2021	China	CD 54 UC 8 IBD-U 4 HC 27	Paediatric	'IBD' 10 (5.3) HC 7.1 (3.8)		Faeces	EZNA soil DNA kit	Amplicon	Illumina MiSeq	V3-4	ND	Shannon Chao1 Sobs	Low
Wang Y et al.	2021	China	CD 22 HC 20	Paediatric		CD 13 (3) HC 12 (2)	Faeces	Qiagen QIAamp stool mini kit	Amplicon	Illumina NovaSeq	V3-4	186189	Shannon Chao1 Simpson Inverse Simpson	Low
Galipeau et al.	2021	Canada	UC 9 FC 48	Adult (?)	UC 19.7 (7.2) FC 20.3 (7.3)		Faeces	Qiagen QIAamp stool mini kit	Amplicon	Illumina MiSeq	V4	12510	Shannon	Low
Juyal et al. *	2021	India	UC 14 HC 36	Adult	ND		Faeces	Qiagen DNeasy PowerLyzer PowerSoil Kit	Amplicon	Illumina MiSeq	V3-4	20000 rarefied	Shannon Chao1	High
El Mouzan et al.	2021	Saudi Arabia	CD 17 SC 18	Paediatric		CD 15 (ND) SC 16.3 (ND)	Mucosal biopsy and faeces	Mobio Powersoil kit	Amplicon	Illumina MiSeq	Other - bTEFAP	At least 1000	Shannon	Low
Paljetak et al.	2022	Croatia	CD 10 UC 13 SC 26	Adult		CD 46 (ND) UC 31 (ND) SC 31 (ND)	Mucosal biopsy	MasterPure DNA purification kit	Amplicon	Illumina MiSeq	V3-4	7916 median	Shannon Chao1 Phylogenetic diversity	Low
Rimmer et al.	2022	England	CD 13 UC 18 HC 18	Adult	ND		Faeces	Qiagen QIAamp stool mini kit	Amplicon	Illumina MiSeq	V4	45079	Shannon	Low
Lv et al.	2023	China	CD 27 HC 27	Paediatric	CD 12.7 (1.6) HC 11.9 (1.8)		Faeces	DP712 kit	Metagenomic	Illumina NovaSeq	ND	ND	Shannon	Low
Total samples included in meta-analysis % samples from paediatric patients	**All sample types** **All IBD 553 (77%)** CD: 411 (92%) UC: 138 (29%) IBD-U: 4 (100%) **All controls 515 (74%)** SC: 267 (70%) HC: 248 (78%) Familial controls excluded						**Faeces** **All IBD 296 (78%)** CD: 240 (91%) UC: 52 (21%) IBD- U: 4 (100%) **All controls 248 (78%)** Only HC data presented Familial controls excluded				**Mucosal biopsies** **All IBD 257 (74%)** CD: 171 (94%) UC: 86 (34%) **All controls 267 (77%)** Only SC data presented Familial controls excluded			
# of variations in methods	**Location: 12** countries of origin **Sample type: 2** (in this analysis, 2 others excluded) **Extraction kit: 12**						**Sequencing type: 2** **Sequencing platform: 4** **Sequence domain (if amplicon): 7**				**Diversity index presented: 11**			

SD = Standard deviation, IQR = Interquartile range, CD = Crohn's Disease, UC = Ulcerative Colitis, HC = Healthy control, SC = Symptomatic control, IBD-U = Inflammatory Bowel Disease unclassified, ND = Data not presented by authors, bTEFAP = bacterial tag-encoded FLX amplicon pyrosequencing, * = Data from preprint only

Analyses were split by sample type. All studies of faecal samples utilised asymptomatic ‘healthy’ controls, whilst all controls were symptomatic in biopsy studies. The synthesis for faecal samples includes a ‘Mixed’ IBD group to allow inclusion of studies where diversity has been published only for IBD overall, rather than by disease subtype. Significant reductions in diversity were seen in IBD relative to HCs across mixed cohorts, CD and UC. This gave a pooled SMD of -1.72 (95% CI -2.45 – -1.05, p<.00001). Nonetheless, high levels of heterogeneity were observed (i^2=89%).The data is presented in ***Figure 1-4.***

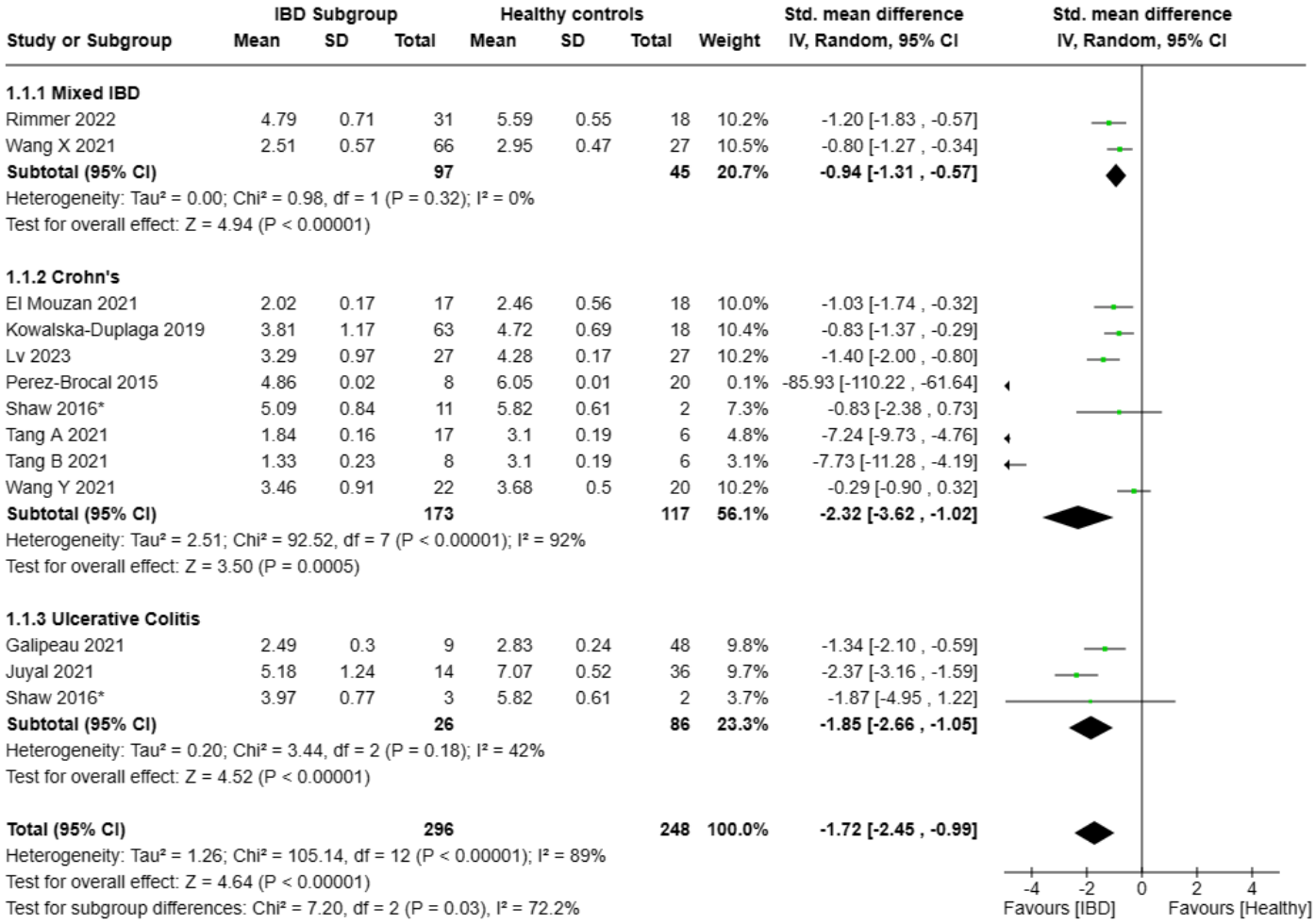


Figure 1 - 4: Meta-analysis and standardised mean difference in Shannon diversity derived from faecal samples between IBD and healthy controls.

Standardised mean difference in Shannon diversity from faecal samples from 296 IBD patients and 248 healthy controls. Significant reductions in diversity were seen in IBD across studies presenting mixed cohorts (IBD n=97, HC n=45; Std. mean diff -0.94 95% CI -1.31 – -0.57), Crohn's disease (CD n=173, HC n=117; Std. mean diff -2.32 95% CI -3.62 – -1.02) and Ulcerative colitis (UC n=26, HC n=86; Std. mean diff -1.85 95% CI -2.66 – -1.05). This gave a pooled standardised mean difference of -1.72 (95% CI -2.45 – -1.05 p<.00001). Nonetheless, high levels of heterogeneity were seen (I^2 =89%). Tang is split into A and B due to the presentation of diversity indices split by subsequent treatment non-responders and responders.

Regarding mucosal biopsies, no studies presented a mixed IBD cohort, as such UC and CD can be presented separately. First, CD was analysed ***(Figure 1-5).*** A trend for slight reductions in diversity in CD did not reach statistical significance (SMD -0.16 95% CI -0.45 – 0.12 p=0.25). Heterogeneity was low (i^2=27%). Next, UC was analysed ***(Figure 1-6).*** No difference was observed in alpha diversity (SMD -0.00 95% CI -0.30 – 0.30 p=0.84). Heterogeneity was low (i^2=0%).

Whilst this CD dataset allows for meaningful comparison, the size of the cohort does speak to the missed opportunities in the way some large studies present data. Several large studies gave no diversity indices. Gevers et al. (2014) included 447 CD patients and 221 symptomatic controls with both mucosal biopsies and faecal samples. Diversity indices appear to have been calculated, and one plot alludes to Chao1, but no retrievable indices are seen. Instead, a novel measure, the 'dysbiosis index' is developed, based on the most abundant taxa seen in CD. Whilst this does correlate with the Paediatric Crohn's Disease Activity Index (PCDAI), it has no precedent and the absence of the core indices on which it is based prevents the utilisation of a huge dataset that would add real value to our meta-analysis.

The smallest cohort was seen in the comparison of mucosal biopsies in UC patients and symptomatic controls. The paucity of data here highlights the issues of reproducibility in microbiome research. There are large and well conducted studies in the area, but publications from the PROTECT cohort (Schirmer et al., 2018 and Hyams et al., 2019) present analyses based on outcomes without controls. Lloyd-Price et al. (2019) includes a mixed cohort of over 100 IBD patients and alludes to

diversity but again the authors create their own concept of ‘dysbiotic episodes’ derived from a score based on beta diversity indices (Bray-Curtis dissimilarities to non-IBD metagenomes) to emphasise differential microbial abundance considered to be furthest from health. Despite this, at no stage is a unit of measure relating to diversity, or a data plot, presented. Morgan et al. (2012) recruited a large adult cohort of IBD patients (121 CD, 75 UC) and analysed both mucosal biopsies and stool samples. However, they included patients within 12 months of diagnosis regardless of prior treatment and did not separate out any data derived from treatment naïve cohorts, completely preventing its inclusion.

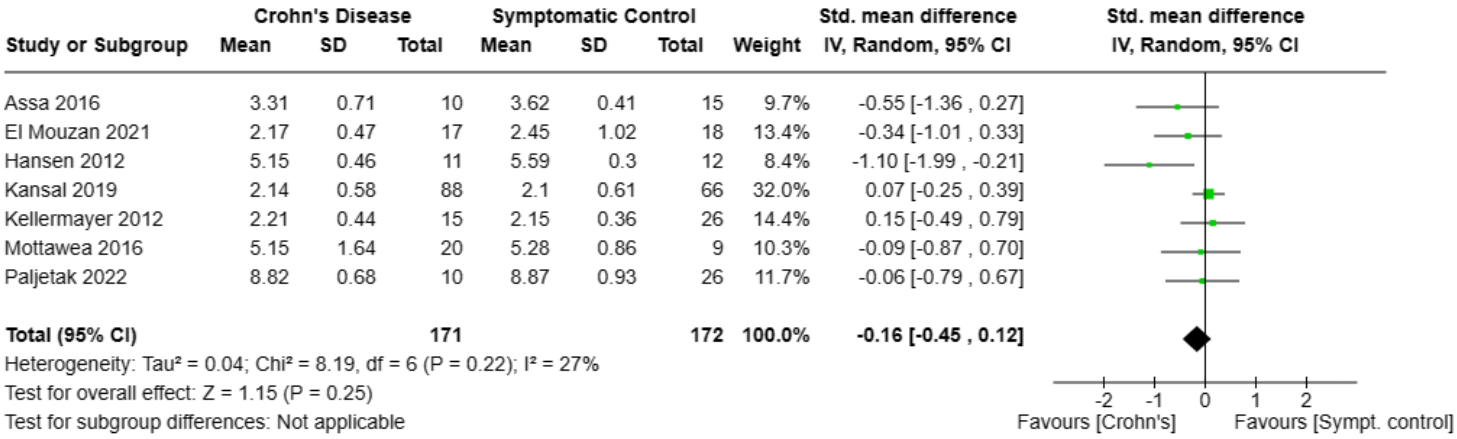


Figure 1 - 5: Meta-analysis and standardised mean difference in Shannon diversity from mucosal biopsies between Crohn's disease and symptomatic controls. This analysis encompassed 171 CD patients and 172 symptomatic controls from 7 studies. Heterogeneity was much lower (I^2 = 27%). Whilst a trend for slight reductions in diversity in CD were observed, these did not reach statistical significance (CD n=171, SC n=173; Std mean diff. -0.16 95% CI -0.45 – 0.12. p=0.25).

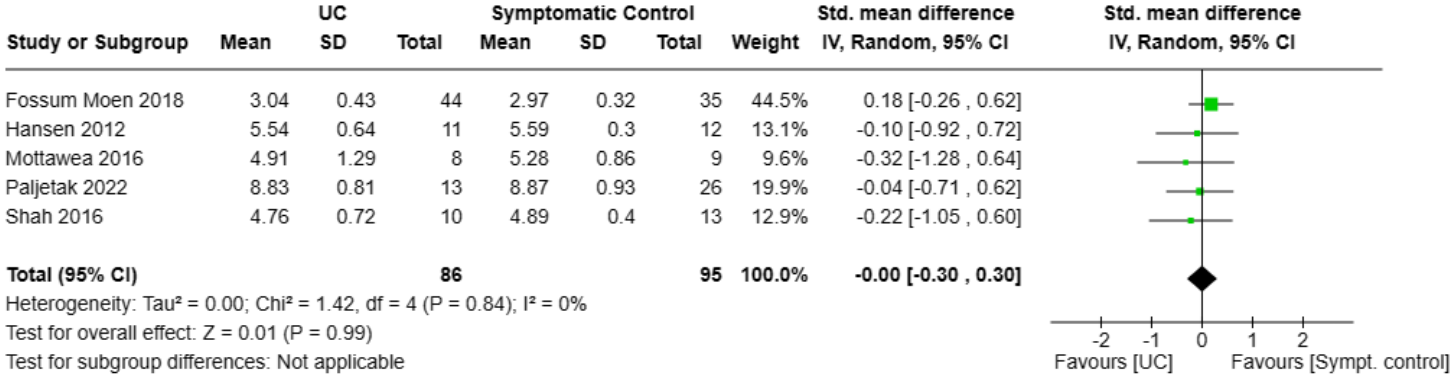


Figure 1 - 6: Meta-analysis and standardised mean difference in Shannon diversity from mucosal biopsies between Crohn's disease and symptomatic controls.

Across a smaller cohort of 86 UC patients and 95 SCs from 5 studies, no difference is seen in alpha diversity (std mean diff. -0.00 95% CI -0.30 – 0.30 p=0.99). This was supported by very low heterogeneity (I^2 = 0%).

Whilst it is apparent that there are significant differences in the faecal stream between IBD and healthy individuals, the size and significance of these differences is not replicated in mucosal biopsies when comparator samples are obtained from symptomatic individuals without IBD. This is particularly true in ulcerative colitis. The documented reductions in diversity associated with IBS, the main constituent of many of these control cohorts, is likely to be highly relevant (Wang et al., 2020). However, given the absence of a comparison between faecal samples from symptomatic individuals, other contributing variables may be overlooked. Certainly, patients with IBD are more likely to have fast intestinal transit and factors such as malabsorption. Do these factors lead to changes in the faecal stream being disproportionate? It is not possible to answer this on the strength on the current data, but I hope with the analyses presented throughout this thesis to be able to further contribute to this argument. One conclusion that can be drawn is that this data strengthens the now established view that additional factors beyond microbial perturbations alone must be present to precipitate IBD.

1.4.2.3 Beta diversity at IBD onset

Beta diversity is typically not presented in a meta-analysable fashion. Indices typically measure dissimilarity or distance between each individual sample comprising two groups. A single presentable number is not generated, and findings are most frequently visually represented with principal coordinate analysis plots or non-metric multidimensional scaling. As such, it is not possible to present a methodologically robust integration of the descriptions of this data. As I look to integrate our sequence data with publicly available datasets later in my thesis I will revisit this.

1.4.2.4 Differential microbial abundance – thematic analysis

A theme of microbiome studies in IBD is the depletion of short-chain fatty acid (SCFA) producing bacteria of the *Firmicutes* phylum, alongside enrichment of genera from the *Proteobacteria* phylum. However, at deeper taxonomic levels, differential signals can be seen amongst species from the same genus. Couple this with the extensive methodological variability across studies and sequencing modalities, head-to-head comparisons of differential microbial abundance are challenging. At higher taxonomic levels, conclusions can be misleading or fail to identify subtle differences.

Here I have undertaken a thematic analysis of all full text papers presenting data from pre-treatment IBD. I aim to set out the status quo regarding differential abundance and identify core themes and important contradictions in the data. Once I have presented my own data later in this thesis I will revisit differential abundance by attempting to integrate this with published raw sequence datasets and undertake repeat analysis through a single bioinformatic pathway. A table listing included studies with basic methodological parameters is in **Appendix A.**

Figure 1 - 7: Word cloud of the 100 most frequently mentioned microbial taxonomic classifications across 60 full text publications presented data on the pre-treatment gastrointestinal microbiome in IBD.

As above, this word cloud presents the 100 most frequently taxonomic classifications across all full text papers included within our systematic review. More frequently appearing terms appear in larger font. At genus level, the most frequently mentioned taxa were *Bacteroides* (299 mentions across 40 studies), *Escherichia.shigella* (223 mentions across 37 studies) and *Faecalibacterium* (214 mentions across 38 studies, though the species *Prausnitzii* was mentioned 271 times in total across 36 papers).

Whilst ***Figure 1-7*** delivers a visual representation, it does not allow any meaningful interpretation or inference regarding the direction of differential abundance or function. To achieve this, the top 25 genera were then analysed across all papers to draw out themes regarding differential abundance, with attention given to species level differences relative to controls, alongside associations with disease activity, treatment outcome and microbial function within these genera. Genera were only included if mentioned on more than 10 occasions across 10 or more papers.

Table 1 - 2: Patterns of differential abundance, relationship to disease activity and functional capacity of the 25 most frequently mentioned bacterial genera across 61 full text publications relating to the pre-treatment gastrointestinal microbiome in IBD.

Phylum_Class_Order_Family_Genus	**Overall no. of mentions**	**No. studies presenting**	**Species reported** (>10 mentions)	**Direction / association** *Select associations across genus and most frequently reported species*
p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Bacteroidiaceae.**g_Bacteroides**	299	40	Fragilis Vulgatus Uniformis Caccae Ovatus Thetaiotaomicron Massiliensis Dorei Faecis	**IBD vs Controls** <u>*At genus level;*</u> - Bibiloni et al. (2006) – Mucosal biopsy, CD and UC. No difference on PCR based analysis. - Paljetak et al. (2022)- Faeces and biopsies, CD and UC. Marginally reduced in IBD (vs IBS controls) - Assa et al. (2016) – Mucosal biopsies, *Bacteroides* overall enriched in CD patients *(B.ovatus* increased in controls) - Tang et al. (2021) – Faeces, *Bacteroides* decreased in CD vs healthy, increased after response to EEN, but not in non-responders - Basha et al. (2022); PCR based but increased *bacteroides* in UC vs controls. Increasing *bacteroides* = more extensive disease <u>*At Species level;*</u> - Gevers et al. (2014); Mucosal biopsies, *Bacteroides* decreased in CD, in particular *B.uniformis*, negative association *bacteroides* and PCDAI - Kansal et al. (2019); Mucosal biopsies, Marginal decreases in *B.vulgatus, B.galacturonicus, B.uniformis* and *B.caccae* in CD. However, higher abundance *of B. vulgatus* at CD first diagnosis and relapse than remission - Rojas-Feria et al. (2018); Faeces, higher abundance of B. *dorei* in controls than CD. - Zitomersky et al. (2013); Increased mucosal abundance of *B.vulgatus* in UC vs control, *B.cellulosilyticus* in CD vs control - Conte et al. (2006) used PCR techniques to show lower abundance of *B.vulgatus* adherent to ileal mucosa in CD vs controls **Disease activity** - Xu et al. (2018); Mucosal biopsies, *Bacteroides* significantly reduced in IBD mucosa after 5-ASA initiation - Wang Y et al. (2021); Faeces, Positive correlation with height / weight , negatively correlated with disease activity indices in CD - Ashton et al. (2017); Faeces, increase in *Bacteroides* abundance as paediatric CD patients move from into remission **Function** - Galipeau et al. (2021); increased elastase activity in stool correlating with increased abundance of *B.vulgatus* in healthy and in those going on to develop UC. 'Pre-UC' patients also had higher gene expression linked to peptidases that were associated with *B.vulgatus.*
P_Proteobacteria.c_Gammaproteobacteria.o_Enterobacterales.f_Enterobacteri	223	37	Escherichia Coli (The term coli is independently mentioned 264	**IBD vs Controls** <u>*At genus level;*</u> - Lloyd-Price et al. (2019); enrichment of *Escherichia* in IBD vs symptomatic controls across multiple integrated samples during previously discussed 'dysbiotic' episodes. - Wang X et al. (2021); enrichment of *Escherichia.Shigella* in stool at paediatric CD onset vs healthy.

aceae.**g_Escherichia.Shigella**			times across 34 papers)	- Wang Y et al. (2021); enrichment of *Escherichia.Shigella* across IBD subtypes vs healthy. Positive associations with CRP and a disease activity index, negative associations with height and weight. - Enrichment of *Escherichia.Shigella* replicated by Sila et al. (2020) and Diederen et al. (2020). The latter study also found that treatment 'non-responders' had a higher abundance of *Escherichia coli* at baseline than responders. <u>*At species level;*</u> - De Meij et al. (2018); Faeces, significant increases of *Escherichia coli* across both paediatric UC and CD vs healthy. **Disease activity** - Hart et al. (2020); Faeces, negative associations between *Escherichia.Shigella* at Hb / Albumin, positive association CRP / FCP. **Function** - Gevers et al. (2014); positive association between the KEGG pathway for pathogenic *E. coli* derived from ileal mucosa and deeper ileal ulceration in those with CD.
p_Firmicutes.c_Clostridia.o_Oscillospirales.f_Ruminococcaceae.**g_Faecalibacterium**	214	38	Prausnitzii (Independently has more mentions than *'Faecalibacterium'* – 271 across 36 papers)	**IBD vs controls** <u>*At genus level*</u> - Rojas-Feria et al. (2018); Depletion of *Faecalibacterium (prausnitzii)* at CD onset in faecal samples vs healthy. - Wang X et al. (2021); Depletion of *Faecalibacterium* across IBD subtypes, replicated by Wang Y et al. (2021) in CD.. <u>*At species level*</u> - Kansal et al. (2022); no significant difference in the abundance of *F. prausnitzii* between CD and controls. - Basha et al. (2022); reduced abundance of *F. prausnitzii* at UC onset, abundance inversely proportional to disease severity. - Perez-Brocal et al. (2015); depletion of *F. prausnitzii* in faecal samples at CD onset. - Gevers et al. (2014) and Kowalska-Duplaga et al. (2018) also mirrored these findings in biopsies and faecal samples respectively. - Lloyd-Price et al. (2019) reported depletion of *F. prausnitzii* in IBD vs controls, particularly during dysbiotic episodes. Shifts in *F. prausnitzii* were more pronounced over longitudinal follow up in IBD than controls. - In one early paper, Hansen et al. (2012) found enrichment of *F. prausnitzii* at CD onset. However, this paper relied on small numbers and statistical methods that were subsequently superseded. - Nonetheless, Assa et al. (2016) also bucked the trend, reporting enrichment of *F. prausnitzii* in mucosal biopsies at CD onset. In UC, Shah et al. (2016) also reported increased abundance in mucosal biopsies vs symptomatic controls (across 6 OTUs for *F. Prausnitzii).* **Disease activity** - Wang X et al. (2021); positive associations between faecal *Faecalibacterium* abundance and height, weight and serum albumin. - Mottawea et al. (2016); depletion of *F. prausnitzii* in more severe disease at CD onset at the mucosa-luminal interface (aspirate obtained after washing the mucous layer off the caecal mucosa during colonoscopy). **Function** - Galipeau et al. (2021); *F. prausnitzii* showed greater functional expression of four gene families related to peptidases when comparing patients who subsequently went on to develop UC with healthy controls.
p__Firmicutes.c__Clostridia.o__Clostridi	195	34	Coccoides	**IBD vs controls** <u>*At genus level*</u> - El Mouzan et al. (2018); mucosal biopsies and faeces, significant depletion of *Clostridium* at CD onset.

ales.f__Clostridiaceae.**g__Clostridium**				- Wang X et al. (2021); overall depletion in *Clostridium,* though there was enrichment of *Clostridium sensu stricto1 (*a separate clostridium genus) in IBD compared to healthy controls (faecal samples). - Wang Y et al. (2021); depletion of *Clostridium clusters IV* and *XIVb.* - Galipeau et al. (2021); enrichment of *Clostridium* in patients who go on to develop UC vs healthy. - Mottawea et al. (2016); enrichment of *Clostridium* at CD onset (mucosa-luminal interface) vs symptomatic controls. - Gevers et al. (2014); *Clostridium* depleted in mucosal biopsies from CD patients vs symptomatic controls. *At species level* - Kansal et al. (2019); Depletion of *C. disporicum, C. maritimum* and *C. lavalense* in CD vs symptomatic controls (mucosal biopsies). CD patients also demonstrated enrichment of *C. butyricans* and *lactatifermentans.* - Rojas-Feria et al. (2018); reported enrichment of *C. bolteae* in CD. - El Mouzan et al. (2018); depletion of *C. disporicum* in colonic biopsies and faeces, *C. perfringens* depleted in ileal biopsies. - Douglas et al. (2018) reported increased levels of a strain of *C. symbiosum* in CD (via random forest modelling, though the cohort was small for this approach). - Kowalska-Duplaga et al. (2019) reported significant depletion of *C. celatum* at CD onset. **Disease activity** - Hart et al. (2020); increases in *C. leptum* following induction therapy with EEN. This correlated with reductions in disease activity. **Function** - Mottawea et al. (2016); significant associations between *Clostridium* and *C. leptum* with mitochondrial protein expression. - Lloyd-Price et al. (2019); increased transcriptional activity of *C. hathewayi* and *bolteae* in IBD during dysbiosis episodes
p_Campilobacterota.c_Campylobacteria.o_Campylobacterales.f_Campylobacteriaceae.**g_Campylobacter**	138	14	Showae	**IBD vs Controls** *At genus level* - Kugathasan et al. (2017); significant enrichment of Campylobacter in mucosal biopsies at CD onset. - Sjöberg et al. (2017); significant depletion of *Campylobacter* in the duodenal fluids of patients with CD vs symptomatic controls. **Disease activity** - Schirmer et al. (2018) reported enrichment of *Campylobacter* in those with more severe disease at UC onset. **Other approaches** - A number of the texts focussing on *Campylobacter* take a different approach. Varma et al. (2022) focus on the number of patients with positive stool PCRs for *Campylobacter* prior to a diagnosis of IBD, whilst papers from Hartley et al. (1992) and Schumacher et al. (1993) are based on techniques not relevant in this analysis. Hansen et al. (2012) also took an alternative approach, subjecting mucosal biopsies from de novo IBD and controls to microaerophilic culture. In this approach, it was not possible to identify strong associations between *Campylobacter* and IBD.
p_Firmicutes.c_Clostridia.o_Oscillospirales.f_Oscillospiraceae.**g_Ruminococcus**	110	26	Gnavus Bromii Torques	**IBD vs Controls** *At genus level* - Paljetak et al. (2022); Faeces, depletion of *Ruminococcus* in IBD patients vs healthy, strongest difference in UC. These differences did not maintain significant in comparisons with symptomatic controls. *At species level* - Rojas-Feria et al. (2018); depletion of *R. albus* in faeces at CD onset vs healthy (as well as depletion in *Ruminococcus* overall). - El Mouzan et al. (2018); faeces, depletion of *Ruminococcus* and *R. flavefaciens* in CD. Not replicated in mucosal biopsies.

				- Gevers et al. (2014); overall depletion of *Ruminococcus,* in particular *R. Bromii* in mucosal biopsies at CD onset. Enrichment of *R. gnavus* reported but not found in supplementary data tables. - Lloyd-Price et al. (2019); enrichment of *R. gnavus* during 'dysbiotic' episodes in CD, with the same pattern seen for *R. torques* in UC. A critique of this approach has been offered in earlier microbial taxa explorations. - Kowalska-Duplaga et al. (2019); depletion of *R. Bromii* in faecal samples at CD onset. **Disease activity** - Hart et al. (2020); in patients receiving induction with EEN or steroids, increase in *Ruminococcus* correlated with increases in Hb. - Wang Y et al. (2021); lower levels of *Ruminococcus* in CD, but also to associating with lower levels of faecal SFCAs in this cohort. - Magnusson et al. (2017); higher levels of *Ruminococcus* at UC onset associated with a subsequent milder disease course. - Kugathasan et al. (2017); increased abundance of *Ruminococcus* in ileal and rectal biopsies from CD patients with stricturing vs uncomplicated (B1) Crohn's disease. - However, Mottawea et al. (2016) found *Ruminococcus* abundance negatively correlated with disease activity indices in CD. - Schirmer et al. (2018); marginal increases in abundance of *R. gnavus* and *torques* at UC onset in association with more severe disease (supplementary table 5) though the MaAsLin data (supplementary table 6) appears discordant with this.
p_Firmicutes.c_Clostridia.o_Lachnospirales.f_Lachnospiraceae.**g_Roseburia**	107	25	Intestinalis	**IBD vs Controls** <u>*At genus level*</u> - Depletion of *Roseburia* in CD mucosal biopsies reported from the RISK cohort (Gevers et al., 2014 and Kugathasan et al., 2017). - Finding replicated vs healthy in stool (Rojas-Feria et al., 2018 and Wang Y et al., 2021) and vs symptomatic controls across both stool and biopsies (El Mouzan et al., 2018). - Paljetak et al. (2022) did not demonstrate significant differences between IBD (either subtype), IBS or healthy (stool or biopsy). - Shah et al. (2016); mucosal biopsies, significant depletion in UC vs symptomatic controls <u>*At species level*</u> - Kansal et al. (2019); depletion of *Roseburia* in CD vs controls. Species level depletion seen in *R. hominis, inulinivorans* and *intestinalis.* Both Kaakoush et al. (2012), Gevers et al. (2014) and Kowalska-Duplaga et al. (2019) found depletion of *R. faecis* in CD. **Disease activity** - Mottawea et al. (2016); negative correlations between *Roseburia* and disease severity - Diederen et al. (2020); In CD, responders to EEN had higher baseline levels of *Roseburia* **Function** - Lloyd-Price et al. (2019); *Roseburia* depleted in IBD, but also linked metagenomically (in particular *inulinivorans* and *intestinalis)* to bile acids and a number of acylcarnitines. - Wang Y et al. (2021); lower levels of faecal SCFAs in patients with lower levels of *Roseburia* (amongst other taxa) in CD.
p_Fusobacteriota.c_Fusobacteriia.o_Fusobacteriales.f_Fusobacteriaceae.**g_Fusobacterium**	101	17	Nucleatum	**IBD vs Controls** <u>*At genus level*</u> - Kansal et al. (2019); significant enrichment of *Fusobacterium* in CD mucosal biopsies. - Paljetak et al. (2022); also reported enrichment, though only significant when comparing faeces UC and healthy. - Gevers et al. (2014); *Fusobacterium* discussed but statistically significant enrichment is only found at family level. <u>*At species level*</u>

				- El Mouzan et al. (2018); increased *F. nucleatum* in faecal samples at CD onset vs symptomatic controls. **Disease activity** - Hart et al. (2020); after completion of induction therapy with either EEN or corticosteroids, abundance of *Fusobacterium* reduced (outside of statistical significance) in both CD and UC. - Mottawea et al. (2016); *Fusobacterium* enriched in CD patients with more severe disease at presentation, as well as showing increased abundance in mucosa-luminal interface samples when compared to symptomatic controls. - Shaw et al. (2016); *Fusobacterium* associated with IBD over controls, as well as higher levels associating with non-response to therapy. However, these signals were not significant after correction for multiple testing. - Gevers et al. (2014); baseline *Fusobacterium* in CD positively correlated with future PDCAI during longitudinal follow up - Schirmer et al. (2018); *Fusobacterium* strongly associated with more severe disease at paediatric UC onset. In this cohort, the abundance of *Fusobacterium* was twice as high in mucosal biopsies than faecal samples from the same patients.
p_Firmicutes.c_Negativicutes.o_Veillonella-Selenomonadales.f_Veillonellaceae.**g_Veillonella**	95	19	Dispar	**IBD vs Controls** <u>*At genus level*</u> - Paljetak et al. (2022); enrichment of *Veillonella* in UC vs healthy. Not significant in CD, or in either group vs symptomatic controls. - Mottawea et al. (2016); enrichment of *Veillonella* in CD vs symptomatic controls, positive associations with disease severity. <u>*At species level*</u> - Gevers et al. (2014); enrichment of *V. dispar* and V. *parvula* in mucosal samples at CD onset vs symptomatic controls. - Perez-Brocal et al. (2015); increased abundance of *V. dispar* in faeces from IBD vs healthy controls. - Kansal et al. (2019); enrichment of *V. atypica* and *V. parvula* in CD mucosal biopsies vs symptomatic controls. **Disease activity** - Hart et al. (2020); depletion of *Veillonella* after either EEN or corticosteroids, though not reaching statistical significance. - Wang X et al. (2021); positive correlations between disease severity and *Veillonella*. - Basha et al. (2022); *Veillonella* abundance directly proportional to disease severity at UC onset. Abundance increased as the colonic involvement became more extensive. - Kugathasan et al. (2017); increased abundance of *Veillonella* in the ileum of CD patients with penetrating complications. - Shaw et al. (2016); increased abundance of *Veillonella* in non-responders, not significant after correction for multiple testing. - Schirmer et al. (2018); increased abundance of *V. dispar* and *parvula* in those with more severe disease at UC onset.
p_Firmicutes.c_Clostridia.o_Lachnospirales.f_Lachnospiraceae.**g_Blautia**	91	21	Obeum	**IBD vs Controls** <u>*At genus level*</u> - Gevers et al. (2014); depletion of *Blautia* from the mucosal biopsies of treatment naïve CD patients vs symptomatic controls. - Wang X et al. (2021); reduced abundance across IBD subtypes vs healthy (faeces). Replicated in CD cohort by Wang Y et al. (2021). - Paljetak et al. (2022); no difference between mucosal biopsies from symptomatic controls and IBD. A significant difference was seen between CD and UC, lower abundance in UC patients. Though not significant, this difference was mirrored in faeces. - In contrast to most papers, Diederen et al. (2020) reported increased abundance in IBD compared to healthy controls. <u>*At species level*</u> - Kansal et al. (2019); reduced abundance of *B. obeum* and *luti* at CD onset in vs symptomatic controls (mucosal biopsies). - De Meij et al. (2018); reduced abundance of *B. producta* and IBD onset vs healthy.

				Function - Galipeau et al. (2021); *B. obeum* showed greater functional expression of four gene families related to peptidases when comparing patients who subsequently went on to develop UC with healthy controls. **Disease activity** - Mottawea et al. (2016) reported negative correlations between disease severity indices and *Blautia.* - Hart et al. (2020); increases in *Blautia* as patients, via steroids or EEN, responded to treatment (across IBD subtypes). - Wang X et al. (2021) and Wang Y et al. (2021); positive correlations between *Blautia* and height/weight. Negative correlations observed with disease activity indices.
p_Bacteroidota.c_Bacteroidia.o_Bacteroidales.f_Prevotellaceae.**g_Prevotella**	82	24	Copri	**IBD vs Controls** <u>*At genus level*</u> - Paljetak et al. (2022); no differential abundance of *Prevotella* in IBD, enrichment in faecal samples from 'IBS' patients vs healthy. - Xu et al. (2018); enrichment of *Prevotella* at inflamed vs non inflamed sites in UC patients. - De Meij et al. (2018); increased abundance of *Prevotella* in healthy individuals compared to IBD patients. - Sila et al. (2020); reported opposite change, increased abundance of *Prevotella* in IBD faeces vs healthy or unaffected siblings. <u>*At species level*</u> - Assa et al. (2016); increased of *P. Copri* in healthy vs CD, replicated by Douglas et al. (2018) in random forest modelling. - Lloyd-Price et al. (2019); no baseline difference in *P. Copri,* but larger abundance shifts in healthy vs IBD over follow up. <u>*Other analyses*</u> - Elmaghrawy et al. (2023); oral microbiome in new IBD, depletion of *Prevotella* in the dorsal tongue reported in IBD vs healthy. **Disease activity** - Wang X et al. (2021); positive correlation with height / weight and negative correlation with disease activity indices. The opposite was described by Mottawea et al. (2016) with positive correlations between disease severity and *Prevotella* abundance.
p_Firmicutes.c_Bacilli.o_Lactobacilli.f_Streptococcaceae.**g_Streptococcus**	82	22	Salivarius	**IBD vs Controls** <u>*At genus level*</u> - El Mouzan et al. (2018); reduced abundance of *Streptococcus* in faeces at CD onset vs symptomatic controls. Analysis mucosal samples revealed a species level depletion of *S. salivarius.* - A pre-print from Juyal et al. (2022) reported the opposite, with increased *Streptococcus* in faeces at UC onset (vs healthy). This was also reported in faecal samples at CD onset vs healthy by Tang et al. (2021). **Disease activity** - Hart et al. (2020); reducing abundance of *Streptococcus* as patients responded to therapy with either corticosteroids or EEN. - Wang X et al. (2021); *Streptococcus* positively associated with disease activity indices at IBD onset. - Magnusson et al. (2017); higher baseline *Streptococcus* associated with a subsequent moderate to severe disease course in UC. - Schirmer et al. (2018) also found significant enrichment of *S. anginosus* in UC patients with severe compared to mild disease. - Mottawea et al. (2016) found positive associations between disease activity and *Streptococcus.*

				- A dissenting view to this trend was provided by Wang Y et al. (2021) who reported higher levels of *Streptococcus* at baseline were associated with sustained response in those subsequently exposed to Infliximab therapy.
Eubacterium* More modern classifications have multiple different Eubacteria genera within the Clostridia class. Earlier classifications did not make this distinction. Key genera reported in the 'species' column.	7	21	Eligens Rectale Coprostanoligenes Halli	**IBD vs Controls** <u>*At genus level*</u> - Kansal et al. (2019); depletion of *E. rectale* and *E. oxidoreducens* in the mucosa of CD vs symptomatic controls. - Rojas-Feria et al. (2018) evaluated CD faeces vs healthy and found depletion of *E. ramulus, eligens* and *coprostanoligenes.* El Mouzan et al. (2018) also mention depletion of *E. seraeum* and *halli* in CD cohorts. - Gevers et al. (2014) reported *E. rectale* as a dominant species reduced at CD onset in mucosal biopsies. Despite this, the supplementary tables only report significant differential abundance for *E. dolichum* and *bioforme.* - Diederen et al. (2020) reported a similar signal for depletion of *Eubacterium rectale* at CD onset. - No papers dealing with UC presented significant differences outside of labelling *Eubacterium* as a single genus. De Meij et al. (2018) and Paljetak et al. (2022) reported depletion of *Eubacterium* in this way, whilst Sjöberg et al. (2017) also reported depletion in the duodenal fluids of treatment naïve UC patients. **Function** - Galipeau et al. (2021); *E. halli* showed greater functional expression of two gene families related to peptidases in patients who went on to develop UC compared to healthy controls. **Disease activity** - Mottawea et al. (2016); *E. rectale* negatively correlated with disease severity in CD and positively associated with mitochondrial protein expression.
p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Bacteroidiaceae.**g_Parabacteroides**	71	20	Distasonis	**IBD vs Controls** <u>*At genus level*</u> - Hansen et al. (2012); depletion of Parabacteroides in the mucosa of UC patients vs symptomatic controls. Rojas-Feria (2018) reported the same pattern in the faeces of newly diagnosed CD vs healthy. <u>*At species level*</u> - Zitomersky et al. (2013); significantly lower abundance of *P. distasonis* at inflamed sites vs mucosa from symptomatic controls. - Kansal et al. (2019); lower abundance of *P. merdae* in mucosal biopsies obtained at CD onset vs symptomatic controls. - Conversely, Galipeau et al. (2021) reported increased abundance of *P. merdae* in UC faeces vs healthy. **Disease activity** - Wang X et al. (2021); reported in the text that *Parabacteroides* positively associated with height and weight, and negatively associated disease activity at CD onset, but associated figures suggest correlations did not meet statistical significance. - The opposite signal was reported by Mottawea et al. (2016); *Parabacteroides* positively associated with disease severity. - Douglas et al. (2018); higher abundance of *Parabacteroides* at baseline in treatment responders relative to non-responders.
p_Firmicutes.c_Bacilli.o_Lactobacillales.f_Enterococcaceae.**g_Enterococcus**	64	18	Faecalis	**IBD vs Controls** <u>*At genus level*</u> - Wang X et al. (2021); faecal *Enterococcus* enriched in newly diagnosed IBD vs healthy. This signal was present in CD cohorts presented by Kowalska-Duplaga et al. (2019), Wang Y et al. (2021) and Tang et al. (2021). **Disease activity** - Kansal et al. (2019) describe an increase in the abundance of *E. casseliflavus* in CD patients reaching remission when compared to those newly diagnosed. Similarly in UC, Jun et al. (2018) describe increased mucosal *Enterococcus* in

				patients with UC who have been treated with 5-ASA and sampled at non-inflamed sites, compared to inflamed areas pre-treatment. - However, Wang X et al. (2021) reported increases in *Enterococcus* positively correlated with inflammatory markers.
p_Firmicutes.c_Bacilli.o_Lactobacillales.f_Lactobacillaceae.**g_Lactobacillus**	63	19		**IBD vs Controls** <u>*At genus level*</u> - Gevers et al. (2014); depletion of *Lactobacillus* in the mucosa of newly diagnosed CD vs controls, but found enrichment of the same genus in faecal samples. - Paljetak et al. (2022); higher abundance of *Lactobacillus* in faeces from IBD patients vs healthy (across IBD subtypes). No significant difference in IBD vs symptomatic controls. Symptomatic patients had higher abundance than healthy individuals. - Sila et al. (2020) also reported reduction of *Lactobacillus* in IBD vs either healthy siblings or unrelated healthy controls. - El Mouzan et al. (2018); depletion of *Lactobacillus* in CD across faeces and ileal biopsies vs symptomatic controls. - Juyal et al. (2021); enrichment of *Lactobacillus* in newly diagnosed UC vs healthy. **Disease activity** - Kansal et al. (2019); enrichment of *L. casei* in CD patients who had obtained remission when compared to newly diagnosed CD.
p_Actinbacteriota.c_Actinobacteria.o_Bifidobacteriales.f_Bifidobacteriaceae.**g_Bifidobacterium**	62	20	Longum Adolescentis	**IBD vs Controls** <u>*At genus level*</u> - Wang X et al. (2021); significant reductions of *Bifidobacterium* in faeces from newly diagnosed IBD patients (82% CD) vs controls. - Juyal et al. (2021) present a contrast to this in their pre-print with enrichment of *Bifidobacterium* reported in UC vs healthy. <u>*At species level*</u> - Gevers et al. (2014) reported depletion of *B. bifidum, longum, adolescentis* and *dentum* at CD onset vs symptomatic controls (mucosal biopsies), though their supplementary data suggests only the genus level difference and the difference in *adolescentis* met more robust statistical significance. - Kowalska-Duplaga et al. (2019) also reported significant depletion of *B. adolescentis* at CD onset. **Disease activity** - Schirmer et al. (2018); depletion of *Bifidobacterium* in moderate or severe UC compared to those with mild disease. - Wang X et al. (2021); *Bifidobacterium* negatively correlated with disease activity, positively correlated weight/height (across IBD). **Function** - Wang Y et al. (2021); lower abundance of *Bifidobacterium* in CD, but also describe reductions of *Bifidobacterium* associating with a decreased unconjugated bile acid pool, as well as a lower unconjugated/conjugated bile acid ratio.
p_Verrucomicrobiota.c_Verrucomicrobiae.o_Verrucomicrobiales.f_Akkermansiaceae.**g_Akkermansia**	60	12	Muciniphila	**IBD vs Controls** <u>*At genus level*</u> - Kugathasan et al. (2017); *Akkermansia* significantly reduced in faeces and ileal mucosa of newly diagnosed CD patients. - These findings were replicated by Douglas et al. (2018), with reduced levels of both the genus *Akkermansia* and the species *A. muciniphila* found to be reduced at CD onset. This was found across 16S and metagenomic sequencing approaches. - Shah et al. (2016); significant depletion of *Akkermansia* in mucosal biopsies at UC onset vs symptomatic controls <u>*At species level*</u> - Kansal et al. (2019); *A. muciniphila* more abundant in symptomatic controls and CD patients in remission vs those at relapse or at first diagnosis. It was least abundant at first diagnosis.

				- De Meij et al. (2018); greater reduction of *A. muciniphila* in UC than CD, with both showing lower abundance than in controls. **Disease activity** - Shaw et al. (2016); Faeces, higher baseline *Akkermansia* in treatment non-responders vs responders across IBD subtypes. - Xu et al. (2018); *Akkermansia* levels significantly reduced after the initiation of 5-ASA treatment in UC. - Magnusson et al. (2017); higher abundance of *Akkermansia* at diagnosis associated with a milder disease course. **Function** - Galipeau et al. (2021); higher abundance of *Akkermansia* associated with reduced elastase activity in (faecal samples).
p_Proteobacteria.c_Gammaproteobacteria.o_Pasteurellales.f_Pasteurellaceae.**g_Haemophilus**	54	14	Parainfluenzae	**IBD vs Controls** <u>*At genus level*</u> - Paljetak et al. (2022); significant enrichment of *Haemophilus* in IBD mucosa vs symptomatic controls, with a much stronger signal seen in UC than CD (significance not maintained in CD in isolation). This pattern was also repeated in faecal profiles, with UC showing significantly higher abundance of *Haemophilus* than healthy individuals, symptomatic controls and CD patients. - Gevers et al. (2014); enrichment of *Haemophilus (*and *H. parainfluenzae)* in the intestinal mucosa of CD patients. - Mottawea et al. (2016); significant enrichment of *Haemophilus* in CD at the mucosa-luminal interface. - Shah et al. (2016); significant enrichment in mucosal biopsies vs symptomatic controls at UC onset <u>*At species level*</u> - Kansal et al. (2019); *H. parainfluenzae* present in 58.5% of newly diagnosed CD vs 39.8% controls. Significant difference in mean abundance (undertaken without true FDR correction). Levels were also higher at relapse and lower in remission in established IBD. **Disease activity** - Gevers et al. (2014); positive associations between PDCAI and the abundance of *Haemophilus* in newly diagnosed CD. - Schirmer et al. (2018); increased *H. parainfluenzae* associated with increased disease severity in UC at baseline and subsequently increased incidence of colectomy. - Basha et al. (2022) also described positive associations between *Haemophilus* and disease severity at UC onset. - Hart et al. (2020); significant reductions in Haemophilus from baseline as a largely newly diagnosed cohort of patients improved with EEN. Negative correlations with haemoglobin and albumin accompanied by positive correlations with CRP and FCP.
p_Firmicutes.c_Clostridia.o_Lachnospirales.f_Lachnospiraceae.**g_Coprococcus**	48	14		**IBD vs Controls** <u>*At genus level*</u> - Gevers et al. (2014); depletion of *Coprococcus* in CD vs symptomatic controls. Kowalska-Duplaga et al. (2019) replicated this. - Paljetak et al. (2022) presented multiple comparisons but only found significant depletion of *Coprococcus* in faeces between CD and symptomatic controls. The depletion of *Coprococcus* was far more prominent in CD than UC. - Across a mixed IBD cohort (though 80% had CD), Shaw et al. (2016) found *Coprococcus* was significantly reduced in IBD vs controls. Further depletion was seen in those that did not respond to treatment vs those that did. <u>*At species level*</u> - Galipeau et al. (2021) found an increased abundance in *C. catus* in UC vs healthy with positive functional associations with gene families relating to a metalloprotease and a peptidase. - Kaakoush et al. (2012); depletion of *C. Eutactus* and *Coprococcus "Clostridium sp.SS2/1"* in CD mucosa vs symptomatic controls.

				- In the Gevers et al. paper (2014) described above, a species level depletion of *C. comes* and *catus* was reported. - Perez-Brocal et al. (2015) report differing signals from within the *Coprococcus* genus. *Coprococcus* was reported to be increased in CD, yet *C. Eutactus* was significantly depleted in CD. This study did present some hard to rationalise findings, including almost no spread in their diversity indices.
p_Bacteroidota.c_Bacteroidia.o_Bacteroidales.f_Rikenellaceae.**g_Alistipes**	48	13	Finegoldii Putredinis	**IBD vs controls** <u>*At genus level*</u> - Rojas-Feria et al. (2018); significantly lower abundances of *Alistipes* in CD than controls. This was also reported at species level, with depletion of *A. putredinis* and *finegoldii.* Douglas et al. (2018) also found *Alistipes* (and *A. putredinis)* to be an important variable in a random forest model separating CD from symptomatic controls, with lower abundance predicting CD. - Lloyd-Price et al. (2019); reduced abundance of *Alistipes* in mucosal biopsies from IBD vs symptomatic controls, with author determined 'dysbiotic' periods characterised by further depletion of *Alistipes.* <u>*At species level*</u> - Reductions of Alistipes in IBD faecal samples were replicated across IBD subtypes by De Meij et al. (2018), with depletion of both *Alistipes finegoldii* and *putredinis.* However, Kowalska-Duplaga et al. (2019) could not replicate these differences with statistical significance.
p_Proteobacteria.c_Gammaproteobacteria.o_Enterobacterales.f_Enterobacteriaceae.**g_Klebsiella**	40	13	Pneumoniae	**IBD vs controls** <u>*At genus level:*</u> - Elmaghrawy et al. (2023); oral microbiome, IBD patients had enrichment of *Klebsiella* on dorsal tongue vs controls. - Kaakoush et al. (2012); *Klebsiella (K. granulomatis and K. variicola)* more frequently identified in IBD than in controls - Similar increases in *Klebsiella* amongst IBD patients were also seen in papers from Conte et al. (2006) and Sila et al. (2020), who presented PCR and T-RFLP based methodologies respectively. **Disease activity** - Xu et al. (2018) found significant enrichment of *Klebsiella* in inflamed mucosa vs non-inflamed within untreated UC patients.
p_Firmicutes.c_Negativicutes.o_Veillonella-Selenomonadales.f_Veillonellaceae.**g_Dialister**	40	12	Invisus	**IBD vs Controls** <u>*At genus level*</u> - Paljetak et al. (2022); significant enrichment of *Dialister* in the mucosal of both CD and UC vs to symptomatic controls. This difference did not retain significant in stool samples, though levels in IBD were significantly higher than healthy individuals. - Kowalska-Duplaga et al. (2019) found the opposite signal with *Dialister* depleted in IBD and enriched in control stool samples. **Disease activity** - Kellermayer et al. (2012); CD with granulomas had significantly lower abundance of *Dialister (Invisus)* than CD without granulomas. - Shaw et al. (2016); *Dialister* the most important genera in a random forest model separating responders and non-responders across IBD subtypes (reduced in non-responders). Doulgas et al. (2018) were able to replicate this finding in a cohort of CD patients. - Xu et al. (2018); significant enrichment of *Dialister* in inflamed mucosa vs non-inflamed within untreated UC patients.
p_Proteobacteria.c_Gammaproteobacteria.o_Burkholderiales.f_Sutterellaceae.**g_Sutterella**	32	11	Wadsworthensis	**IBD vs Controls** <u>*At genus level*</u> - Kellermayer et al, (2012); increased abundance of *Sutterella* in CD mucosa relative to symptomatic controls. - Paljetak et al. (2022) replicated this difference in CD but found these differences did not remain significant when comparing the faecal profiles of the same patients. IBD patients did have significantly more Sutterella in across subtypes vs healthy controls.

				At species level - Hansen et al. (2012);mucosal biopsies, targeted PCR for *S. wadsworthensis* found no difference in IBD vs symptomatic controls **Disease activity** - Mottawea et al. (2016); Sutterella positively correlated with disease severity in CD. - Douglas et al. (2018); Higher levels of *S. wadsworthensis* predicted CD treatment non-response in a random forest model. - Two large North American studies presenting metagenomics from mucosal biopsies found that lower baseline *Sutterella* associated with higher levels of 52-week corticosteroid free remission in CD (Gevers et al., 2014) and UC (Hyams et al., 2019).
p_Firmicutes.c_Clostridia.o_Lachnospirales.f_Lachnospiraceae.**g_Anaerostipes**	24	10		**IBD vs Controls** *At genus level* - Perez-Brocal et al. (2015); reported, via an LDA cladogram, that *Anaerostipes* was more abundant in CD vs healthy. - However, Kowalska-Duplaga et al. (2019) and Paljetak et al. (2022) reported the opposite signal, with significantly decreased faecal *Anaerostipes* in both CD and UC vs healthy. Paljetak found no differences between IBD and symptomatic controls. **Disease activity** - Tang et al. (2021) reported increases in faecal *Anaerostipes* from baseline in those with CD who responded to EEN

This is a large dataset, with most data limited to description. At times studies are directly contradictory and it is challenging to draw obvious conclusions. This is exacerbated by methodological variations across control groups, sample types and sequencing approaches. An overall theme for depletion of SCFA producing genera, typically from the *Lachnospiraceae* family, and enrichment of genera from the *proteobacteria* phylum is seen. For example, enrichment of *Haemophilus, Escherichia.Shigella, Veillonella, Fusobacterium* and *Sutterella* are associated with IBD over controls in all studies, whilst increased abundance typically is observed to correlate with increased disease severity and worse treatment outcomes. Enrichment of *Roseburia, Bifidobacterium* (apart from one pre-print), *Alistipes* and several *Eubacteria* (particularly *Eubacterium rectale)* associate with health. Several studies present two control groups, one healthy and one symptomatic, and at times report significant differences between these. *Faecalibacterium* (especially *F. prausnitzii)* is the subject of extensive discussion, alongside *Akkermansia* (largely isolated to *A. muciniphila).* Both are generally accepted within the literature to be associated with health. Whilst general trends in these studies would support this, contrasting signals are presented by some, particularly regarding *F. Prausnitzii.*

Microbial function and the links between taxa and different 'omes' are explored in several texts. Mottawea et al. (2016) attempted to analyse the mucosa-lumen interface by washing and aspirating the mucous layer during colonoscopy, in addition to biopsies. These materials were subjected to a number of techniques including 16S rRNA sequencing and proteomics. The authors found the OTUs with the greatest reduction in abundance were those with the strongest positive correlations

with mitochondrial proteins known to be associated with butyrate production. Those with increased abundance were those with negative correlations with mitochondrial proteins including *Atopobium parvulum, Bacteroides, Parabacteroides distasonis and Fusobacterium.* This group displayed negative correlations with up to 31 mitochondrial proteins which included those involved with the H2S-detoxification complex. These hydrogen sulphide pathways are associated with impaired oxidation of fatty acids including butyrate and has been implicated in the aetiology of IBD (Rowan et al., 2009). Lloyd-Price et al. (2019) found metabolite pools to be significantly less diverse in IBD and SCFA such as butyrate, propionate and valerate were reduced. Primary bile acids were enriched and secondary bile acids such as lithocholate and deoxycholate were reduced in dysbiotic samples. Furthermore, inflamed locations harboured differentially expressed genes directly involved with commensal organisms such as CXCL6 and SAA2. Having such a wealth of multi-omic data, it was possible to identify sub-networks linking key depleted taxa, in particular *Subdoligranulum, Faecalibacterium* and *Roseburia* which covaried with cholesterol and inosine. In a secondary analysis of this cohort by Priya et al. (2022), the authors utilised machine learning to identify 25 IBD specific host pathways that associated with gut microbes including the integrin beta-1 pathway which relates to leucocyte recruitment and was seen to be associated with taxa including *Phascolarctobacterium, Peptostreptococcaceae* and *Dialister* amongst others. Wang Y et al. (2021) reported lower levels of faecal SCFAs in patients with lower levels of *Roseburia and Bifidobacterium* (amongst other taxa) at CD onset. Depletion of *Bifidobacterium* was associated in CD with a decreased unconjugated bile acid pool, as well as a lower unconjugated/conjugated bile acid ratio. Within the last 12 months, a Maltese cohort (Rausch et al., 2023) have shown similar patterns in diversity and

taxa abundance but identified added functions, with increased abundance of glutathione reductase, implicated in oxidative stress resistance, and ethanolamine ammonia-lyase, responsible for crucial steps in glycerophospholipid synthesis.

The final adult study that, albeit one not meeting criteria for the review, warrants discussion originates from the same OSCCAR cohort (Shapiro et al., 2021). In this study, IgA-SEQ was undertaken in addition to 16S rRNA sequencing in recently diagnosed patients. Faecal samples, after several lysis and suspension steps were mixed with PE-conjugated Anti-Human IgA. After incubation and washes, fluorescence-activated cell sorting was undertaken for 500.000 IgA positive and negative bacteria. Using this technique, it was possible to identify 43 bacterial taxa with significantly higher IgA coating. Levels of coating were then compared against overall abundance. Increased coating and abundance were seen with taxa including *Haemophilus parainfluenzae.* Taxa previously associated with IBD onset, including *Sutterella* and *Ruminococcus Gnavus* were highly coated in specific individuals but not significantly more coated in IBD than controls as a group. It was suggested that this may owe to some unique strains being more highly immunogenic or genetic factors between individuals.

1.4.2.5 Bacteria don’t paint the whole picture

Much of the focus within IBD circles has been on the bacterial component of the microbiome and certainly that is where I have focussed so far. A secondary paper generated from patients recruited from the PROTECT cohort also focussed on the virome (Tokarz et al., 2019). The faecal virome of 70 UC patients was sequenced

using the VirCapSeq-Vert platform. Higher incidences anelloviruses were seen in patients with active UC, though it was not possible to link any of these to disease severity or outcome.

In addition to studies of the virome, the fungal mycobiome has also been associated with IBD onset. We have already mentioned the paper by Kellermayer et al. (2012). In this study of new onset CD, fungal small subunit sequencing was undertaken alongside bacterial sequencing and a novel association between one fungal genus, *Malassezia*, and granulomatous CD was described. Three other papers have sought to evaluate the role of Fungi in IBD onset. The link with CD was further explored in a small cohort of 15 children with CD (El Mouzan et al., 2017). Faecal samples and mucosal biopsies were analysed using ITS2 sequencing. Although there were no significant changes in overall diversity compared to controls, multiple significant community changes were seen with CD characterised by increases in *Psathyrellaceae* and *Cortinariaceae* families, with significant increases in *Psathyrella artemisiae* seen at a species level (FDR 0.005). Mukhopadhya et al. (2015) utilised 18S rRNA sequencing on mucosal biopsies from 25 paediatric patients (13 CD and 12 UC). In this small cohort, a predominance of the fungal species *Ascomycota* was seen in health, whilst *Basidiomycota* was seen to predominate in IBD. The final paper specifically in UC patients, with a sub-cohort of 20 treatment naïve patients analysed (Xu et al., 2019). ITS1-2 rDNA amplification and sequencing were undertaken on mucosal biopsies. Inflamed mucosa had higher richness but lower evenness than non-inflamed. Inflamed mucosa was characterised by a reduced abundance of *Ascomycota* and increased abundance of the genera *Scytalidium, Sporidiobolus, Vanrija* and *Verticillium*.

1.4.3 Discussion: Where does this leave our understanding of the Inception microbiome?

The gut microbiome has been explored in depth at the onset of paediatric IBD with large scale multi-omic studies. In adult cohorts, the area is less well explored. Methodological inconsistency and the absence of core microbiome indices during the presentation of large inception cohorts limits the strength of the conclusions it is possible to draw.

There is clear separation between overall alpha diversity in pre-treatment faecal samples obtained from IBD when compared to HC. In comparisons using mucosal biopsies from SC, significant differences do not remain. Severe large studies utilising mucosal biopsies, such as Gevers et al. (2014) and Lloyd-Price et al. (2019) omit core indices and present novel measures without providing the core metrics to justify their use.

Regarding differential abundance, core themes do still run through the data. Depletion of taxa from the *Firmicutes* phylum associated with carbohydrate metabolism and SCFA production are a hallmark of new onset IBD, though there are exceptions. Increased abundance of *Fusobacteria* and *Proteobacteria* including *Enterobacteriaceae, Haemophilus, Sutterella* and *Fusobacterium* are consistently described.

Simply demonstrating the presence of given taxa is not adequate to demonstrate cause. Functional analyses possible using metagenomic datasets and multiomic studies characterising the proteome and metabolome have granted greater

understanding of complex microbial functions. Key metabolic pathways, including decreases in butanoate and propanoate metabolism are associated in reduction in key SCFA such as butyrate that are subsequently utilised by colonocytes for key functions such as maintaining the mucous layer as an effective barrier. Alterations in other pathways at IBD onset have been associated with decreased amino acid biosynthesis and environmental alterations favourable to oxidate stress. We know that host-immune interactions are bidirectional and there is clear evidence that adherent pathobionts such as *Escherichia* and *Fusobacterium* may favour the inflammatory environment and drive inflammation. Studies evaluating immune interactions with commensal bacteria have also demonstrated that some strains may be more immunogenic than others.

Whilst it is recognised that inflammation itself will continue drive fluctuation and instability of the microbiome; by focussing on the disease at diagnosis we are able to evaluate the microbial changes in the form most closely resembling the first onset of the condition. We aim with our dataset to increase understanding of the microbial differences seen in the faecal stream between IBD and symptomatic non-IBD, prior to the initiation of treatment. Through integration of our data with publicly available datasets we may be able to offer a more definitive perspective on the role of diversity and the key microbial taxa that are implicated in IBD. Furthermore, we hope to couple our analyses with novel angles on host-immune interactions to further develop our understanding of how disease onset plays out.

1.5 ‘Galectins’ – what are they?

Glycans are carbohydrate-based polymers made by all living organisms. They are amongst the most structurally diverse molecules found in nature. They are essential molecules that have a role in nearly every biological process (Hart, 2023). They are typically found on the extracellular surface of cells, binding via cellular proteins and lipids. Recognition of specific cellular glycans by glycan binding proteins (GBPs), or lectins, can exert a huge array of actions upon a cell. This is dependent upon not only the nature of the specific glycan but also the specific cell, particularly its differentiation and degree of activation (Cummings, 2009). Different glycoconjugates (glycosylated proteins and lipids) have the potential to present the same glycan structure and accordingly GBPs may bind distinct receptor types and result in distinct cellular modulations (Liu and Stowell, 2023). Whilst the functions are clearly diverse, in recent years there has been rapidly growing interest in the role of glycans as key contributors to the regulation of innate and adaptive immune responses (Pereira et al., 2018).

β-galactoside binding lectins, or galectins, are a family of GBPs which were among the first to be studied (Barondes et al., 1994) and the first to be linked with immune function and inflammation. Their carbohydrate recognition domains are responsible for the identification of glycans containing β-galactoside on both proteins and lipids (Nabi et al., 2015). They are synthesised as cytosolic proteins, only reaching their ligands after non-classical secretion bypassing the Golgi complex (Johannes et al., 2018). Once they are secreted into the extracellular compartment, they can exhibit polyvalent interactions with cell surface glycans, including those found on a multitude

of immune cells. However, this does not mean they do not perform intracellular functions, with established evidence for participation in signalling pathways, cell differentiation, autophagy and apoptosis (Liu and Rabinovich, 2010).

To date, more than 16 members of the galectin family have been characterised in vertebrates (Liu and Stowell, 2023). Only 12 have established discovered genes encoding for them within humans (Brinchmann et al., 2018). They are typically grouped according to their structure, which is summarised in ***Figure 1 – 8***.

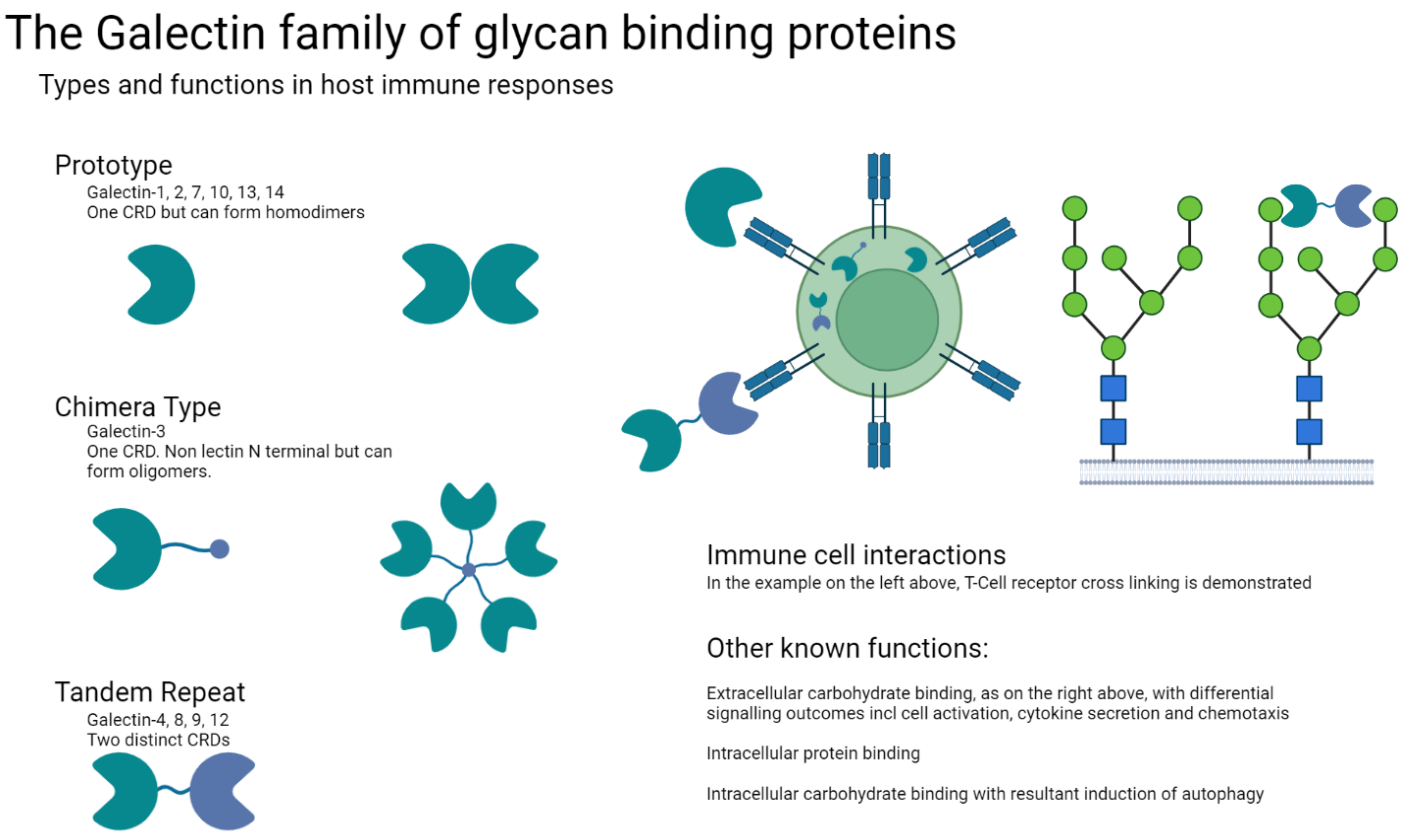


Figure 1 - 8: The galectin family: Structural types and summary of functions

This figure, adapted from Liu and Stowell (2023) and Lightfoot et al. (2021), demonstrates the three structures found amongst galectins. Prototype, chimera type and tandem repeat galectins are described. Oligomerisation of galectin monomers is usually required for the formation of functional galectin-glycan matrices. Different galectins are associated with an array of functions with expression in different tissues. These functions are both intra and extracellular. The only galectin recognised in humans not included here is galectin (GAL) -16. More recently discovered, though also a monomer, it differs significantly from other prototype galectins in its binding and its structure is not fully understood (Si et al., 2021). This figure was created in Biorender. CRD = Carbohydrate recognition domain.

1.5.1 What are the functions of galectins relevant to the pathogenesis of IBD?

In the context of IBD, a number of these roles in immune response are of great interest. Recently summarised in depth (Liu and Stowell, 2023), the points of most interest can be considered in three domains.

The first focuses on the roles of galectins in adaptive immunity and T-cell function. Galectin (GAL) -3 has been shown, extracellularly, to mediate cell migration, adhesion and cell-cell interactions. It can modulate T-cell activation via interactions with extracellular glycans e.g. on CD8. These effects on CD8 in turn weaken T-cell receptor signalling. Influences have also been demonstrated on both T-cell differentiation and contraction. However, it is predominantly cytoplasmic, and its chimeric structure allows it to interact with an array of ligands and carry a broad range of functions (Funasaka et al., 2016). Within the cytoplasm it demonstrates anti-apoptotic activity and can promote non-canonical inflammasome activation through binding lipopolysaccharide glycans (Lo et al., 2021).

GAL-9 is a ligand for T-cell immunoglobulin and mucin domain 3 (Tim3). Tim3 is expressed by terminally differentiated Th1 cells. In vitro, GAL-9 binding Tim3 has been shown to induce T-cell apoptosis and clonal contraction of effector Th1 cells (Kashio et al., 2003 and Zhu et al., 2005). Subsequent mouse studies have demonstrated that over-expression of GAL-9 was associated with significant expansion of $CD11b^{+}Ly\text{-}6G^{+}F4/80^{low}$ cells. Expansion of these cells resulted in significantly lower proliferative T-cell responses in these mice relatively to wild type littermates, alongside almost absent IFN-γ production and increased IL-4 and IL-10

production. Hence this interaction between GAL-9/Tim-3 is postulated to be central to regulation of Th1 mediated immunity (Dardalhon et al., 2010). Oomizu et al. (2012) found that in GAL-9$^{-/-}$ mice, inflammation was exacerbated with expansion of IL-17 producing Th17 cells, alongside contraction of Foxp3+ Tregs. Subsequent in-vitro experiments demonstrated GAL-9 suppressed Th17 whilst increasing Foxp3+ Tregs, which themselves differentiated from naïve CD4 T-cells through a pathway mediated by IL-2. This, in turn, suggests a role for galectin-9 in supressing Th17 development.

Finally, galectin-3 signalling in B cells has been shown to control germinal centre formation, key sites in antibody induction (Beccaria et al., 2018), whilst GAL-9 can influence B cell activation (Giovannone et al., 2018). In mouse models of colitis, GAL-1 administration has been shown to reduce the number of activated T-cells and the production of cytokines (Santucci et al., 2003).

The second focus is the role of galectins in the recruitment of innate immune cells. Leucocyte recruitment is in part modulated by galectins. In psoriatic skin lesions, GAL-3 has been shown to be downregulated within the epidermis (Shi et al., 2018). These authors proceeded, via in vitro experiments and studies in GAL-3 $^{-/-}$ mice, to demonstrate the leucocyte attracting capacity of keratinocytes was greatly increased by impaired expression of GAL-3. Expression of antimicrobial peptides and chemokine ligands also increased. In GAL-3 $^{-/-}$ mice, administration of recombinant GAL-3 could alleviate skin inflammation induced by imiquimod. In mouse models of

airway inflammation induced by streptococcus pneumoniae, GAL-3 knockout mice exhibited compromised recruitment of neutrophils (Nieminen et al., 2008). In mouse models of allergic airway disease, GAL-3 knockouts had reduced eosinophil infiltration, whilst GAL-1 deficiency was associated with airway hyper-responsiveness and eosinophil infiltration (Zuberi et al., 2004; Ge et al., 2010). Innate immune responses related to macrophages are also influenced by galectins. NLR family Pyrin domain containing 3 (NLRP3) is a protein predominantly expressed in macrophages with a central role in inflammasome activation (Kelley at al., 2019). In a mouse model of dextran-sulphate sodium colitis, macrophage activation appeared reduced in GAL-3 knockout mice. Indeed, several studies have demonstrated NLRP3 inflammasome activation within macrophages being promoted by GAL-3 (Markovic et al., 2016). Conversely, interactions between intracellular GAL-9 and NLRP3 were shown to assist the degradation of NLRP3 with GAL-9 knockout mice showing increased NLRP3 dependant inflammation (Wang et al., 2021). Despite this, Intracellular GAL-9 has also been observed to increase phagocytosis (Cano et al., 2019). Across in vitro experiments and in GAL-9 kickout mice, a role for soluble GAL-9 in direct capture / activation of neutrophils, alongside neutrophil adhesion when immobilised, has been demonstrated (Iqbal et al., 2021).

Finally, galectins have been shown to directly engage pathogens, with the ability to regulate host immune responses via several different mechanisms. In vitro, microbial engagement by GAL-3, -4, -8 and -9 resulted in microbial death (Stowell et al., 2014). From a gastrointestinal standpoint, interactions between GAL-3 and Helicobacter Pylori have been demonstrated on the luminal side of the epithelia. In

one mouse model, GAL-3 knockout mice had a higher bacterial load with impaired macrophage function and neutrophil recruitment following infection with Helicobacter (Park et al., 2016). GAL-9 has been shown to opsonise gram-negative bacteria and promote antibacterial immune defence (Schlichtner et al., 2021). Across in vitro and mouse experiments, Jayaraman et al. (2010) have also highlighted the importance of the interaction between Tim3 and GAL-9 in this regard. Expression of GAL-9 by mycobacterium TB infected macrophages, and consequent binding with GAL-9 restricts intracellular bacterial growth and leads to both macrophage activation and IL-1β secretion mediated bactericidal activity. Furthermore, the gene encoding for GAL-9, LGALS9, has already been identified as part of a cluster of risk genes near to NOD2 that are all implicated in the response to mycobacterium tuberculosis, and all confer an increased risk of Inflammatory Bowel disease (Jostins et al., 2012). The functions of GAL-9 are summarised in ***Figure 1-9***.

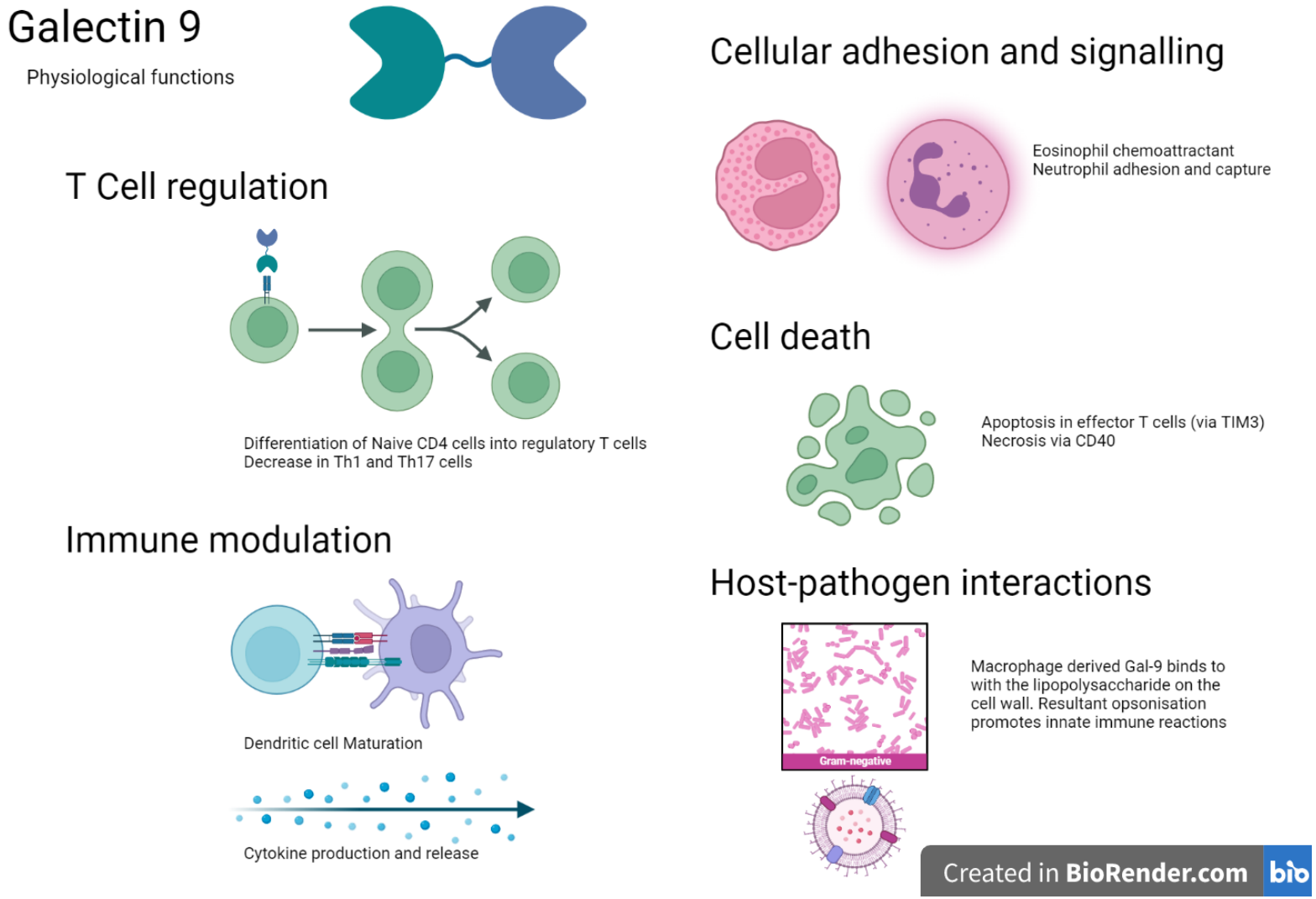


Figure 1 - 9: The roles of GAL-9 in Health and Disease:

Adapted and amended from Moar and Tandon (2021). In vitro, GAL-9 has been shown to have many important functions in the regulation of immune responses and interactions with pathogens. These diverse roles in immune pathways make it an important area for further study in the context of IBD.

Given the complex interactions between host immunity and the gut microbiome present at the onset of IBD, these functions would make galectins highly appealing for further study. This particularly true to those that are abundant in the gastrointestinal tract in humans. GAL-3 and -9 are abundant in many tissues including the gastrointestinal tract, but gastrointestinal expression is also associated with GAL-1, -2, -4, -6 and -7, whilst GAL-10 is highly prevalent in eosinophils (Johannes et al., 2018). Many of these roles are based on work undertaken in vitro or in mouse models, which whilst relevant in the study of galectins, do not represent the development of diseases, without artificial induction, in humans. To understand this, I have undertaken a literature review regarding what is already known about the role of galectins in IBD.

1.5.2 The galectins in Inflammatory Bowel Disease: A literature review

Medline searches were undertaken for using the terms ‘galectins’, ‘Inflammatory Bowel Disease’, ‘Ulcerative Colitis’ and ‘Crohn’s disease’. Only original research studies involving human patients with a clearly defined diagnosis of IBD were considered. A total of 58 studies were identified, which after filtering based on the above criteria, was reduced to 22. Each of these studies has been summarised in Table 1-1. The studies represent a heterogenous group both in methodology and patient cohorts. GAL-3 was the most studied of the galectins, but significant interest was also shown in GAL-1, GAL-3BP, GAL-9 and its binding partner T-cell immunoglobulin and mucin protein 3 (Tim-3). Studies have focussed on both serum levels but also mucosal expression.

From the available evidence in human studies, GAL-3 appears to display contrasting behaviours. Serum levels have been shown to be elevated in IBD relative to controls across several studies (Yu et al., 2020; Fro'lova et al., 2009) yet levels are reduced in inflamed mucosa (Muller et al., 2006; Shkoda et al., 2007; Lippert et al., 2008). Furthermore, serum levels are seen to be highest in those with milder disease and negatively correlate with disease severity (Volarevic et al., 2019; Jensen-Jarolim et al., 2001). Ex vivo stimulation of colonic epithelial cells with TNF led to downregulation of GAL-3 expression. GAL-3 binding protein has also more recently shown promise with Cibor et al. (2016) showing increased levels in active CD relatively to healthy controls. Pesole et al. (2023) followed on from this work by demonstrating that GAL-3BP was associated with an increased risk of both non-response and anti-drug antibody formation when treated with infliximab. GAL-1 also shows mixed behaviours across various studies. Yu et al. (2020) compared 168 IBD patients (97 CD, 71 UC) with 40 healthy controls and found increased GAL-1 levels in the serum of patients with IBD. Amongst 50 IBD patients (40 UC 10 CD) Kessel et al. (2021) found that increased GAL-1 at baseline was associated with an increased risk of relapse over follow up. However, Iwatani et al. (2020) found no difference in serum levels between 53 UC patients and 33 healthy controls. A similarly mixed picture is seen at a mucosal level. Papa-Gobbi et al. (2016, 2021) repeatedly found significantly increased GAL-1 mRNA levels in inflamed colonic mucosa whilst Iwatani et al. (2020) found the opposite despite using similar techniques. GAL-9 has often been studied in the context of its relationship with Tim-3, a known binding partner. However, this has been undertaken in somewhat less depth than the previous galectins, which is in part surprising given its role in host-microbial interactions and the establishment of LGALS9 as a risk gene for IBD. Shi et al. (2012) provided

almost no information about the UC cohort they were studying but found significantly lower GAL-9 mRNA in both PBMCs and mucosal biopsies in their UC patients. Morimoto et al. (2011) found no differences in mucosal GAL-9 mRNA levels, whilst Cibor et al. (2016) found no differences in serum.

The one study undertaken in new onset (and pre-treatment) IBD, albeit as a secondary analysis, found increased LGALS9 transcripts in inflamed mucosa with positive correlations with disease activity (Chen et al., 2020). Indeed, all other studies are characterised by heterogenous cohorts with mixed treatment histories. Attempts to account for variations in therapy, particularly in studies from the biologic era, are limited. This may in part explain the mixed patterns seen in the results and is an important factor to overcome when designing a meaningful evaluation of galectins. No study has tried, in an in vivo manner, to try to link galectin levels with the gut microbiome. Given the role of galectins, in particular GAL-9, in host-immune interactions, this is a highly appealing area of study. Wang et al. (2019) attempted to evaluate this in-vitro. Stool and serum from 20 patients with active UC were collected. The stool was pelleted and suspended in phosphate buffered saline. The faecal bacteria were co-cultured with matched monocytes and followed with FACS and ELISA analysis for markers of activation and inflammatory mediator production assessment. It was found that monocytes from UC patients at baseline had lower Tim-3 expression than controls. When stimulated by autologous faecal bacteria, a higher level of Tim-3 downregulation was also seen in UC samples. After stimulation with faecal bacteria, exogenous soluble GAL-9 was added, and cytokines measured. GAL-9 was less able to suppress the expression of TNF in UC patients relative to

healthy controls. Given the dysregulated immune responses that characterise IBD, impaired or absent immune regulation from Tim3 – GAL-9 interactions, particularly amongst monocytes, could have an important role both in IBD onset and treatment response, though the ex-vivo nature of the microbial component of this study is a clear limitation.

Table 1 - 3: Summary of studies directly evaluating the role of galectins in IBD

Author	Date	Patient population	Prior treatment	Galectins evaluated	Methodology (human samples only)	Key findings (relating to galectins)
Jensen-Jarolim et al.	2001	60 Crohn's, 13 UC, 20 PBC, 5 AIH, 33 Controls	Data not provided	GAL-3	Levels of Anti-GAL-3 antibodies in sera assessed via immunoblotting with recombinant human GAL-3 Anti-GAL-3 derived from CD patients was compared to those derived from various groups. Epitope specificity was characterised using artificial epitopes of GAL-3 using random peptide phage libraries.	Increased proportion of sera from those with CD had anti-GAL-3 antibodies compared to all other groups. CD patients with lower disease activity scores had higher titres and incidences of anti-GAL-3 antibodies reactivity
Jensen-Jarolim et al.	2002	10 CD, 3 UC, 9 Colorectal cancer, 1 non-specific GI inf, 1 Diverticulosis	Not provided	GAL-3	GAL-3 presence in colonic epithelium analysed by Immunohistochemistry, immunoblotting, immunofluorescence and reverse transcription polymerase chain reaction (RT-PCR). Epithelial cell lines and cultured intestinal epithelial cells exposed to cytokines to assess the impact on GAL-3 expression, again via RT-PCR	Homogenous distribution of GAL-3 in controls. However, heterogenous spotting straining in CD lesions and downregulation of GAL-3 expression in inflamed tissue. TNF alpha reduced expression of GAL-3 but not the other cytokines that were tested.
Muller et al.	2006	48 Crohn's, 26 UC, 21 Controls, Healthy volunteers (PBMCs only)	Yes – mixed cohort incl biologics	GAL-3	Mucosal biopsies, PCR of total RNA and Immunohistochemistry Peripheral blood mononuclear cells from whole blood, stimulated in vitro	Comparable expression of GAL-3 in non-IBD controls and IBD in remission. However, significant downregulation of GAL-3 in inflamed mucosa. Ex-vivo stimulation of uninflamed biopsies with TNF led to similar GAL-3 mRNA downregulation as inflammation did in vivo.
Shkoda et al.	2007	6 CD, 6 UC, 6 Cancer resections as non-inflamed controls	Not provided	GAL-3	Ileal or colonic tissue from surgical resections. - Primary intestinal epithelial cells isolated - 2D gel electrophoresis, Matrix assisted laser desorption / ionisation (MALDI-TOF) mass spectrometry and Western blot analysis	Amongst many other findings, significant upregulation of GAL-3 was seen in UC relative to healthy controls
Lippert et al.	2008	21 CD, 7 UC, 13 Colorectal cancer, 3 diverticulitis	Not provided	GAL-3	Surgical resection specimens - Isolation of epithelial cells and fibroblasts - Immunohistochemistry, Immunofluorescence	Reduced expression of GAL-3 in terminal ileal vs colonic mucosa

					- Enzyme linked immunosorbent assays (ELISAs) - Western blots - RT-PCR	Reduced GAL-3 mRNA and protein expression in Crohn's fistulae and stenoses
Brazowski et al.	2009	39 patients with an IPAA for following subtotal colectomy for UC	All post-surgical, patients grouped according to presence and type of pouchitis.	GAL-3	Mucosal biopsies - Immunohistostaining (GAL-3, CD68, SMA abs) - Microscopic evaluation	Significantly reduced staining for GAL-3 in sub-epithelial macrophages in those with chronic or recurrent acute pouchitis.
Frol'ová et al.	2009	58 UC, 68 CD, 71 Controls	Mixed treatment including immunomodulators and biologics	GAL-3	Serum: ELISAs and Western blots Mucosal biopsies: Immunocytochemistry	Increased concentration of GAL-3 in those with active disease vs remission and in those with IBD vs controls. GAL-3 was largely present on enterocytes in controls, but in those with IBD, strong expression of GAL-3 was instead seen on CD-14 positive cells.
Morimoto et al.	2011	Different numbers per specimen type, not disclosed if cross over. Approx 41 CD, 23 UC, 6 controls	Mixed post treatment cohort	TIM3 and GAL-9	Peripheral blood, surgical specimens and mucosal biopsies - Flow cytometry - RT-PCR	TIM3 was constitutively expressed on Th cells from IBD and healthy intestinal mucosa, but at significantly lower levels in those with CD than healthy controls. There were no significant differences in mucosal GAL-9 mRNA levels across groups.
Zhao et al.	2011	12 UC, 12 Controls	Not provided	GAL-3	Mucosal biopsies: 2D electrophoresis, MALDI-TOF, Western blotting, Immunohistochemistry	GAL-3 significantly down regulated in UC as determined by MALDI-TOF. This down regulation was increased in those with severe disease.
Shi et al.	2012	This paper makes no reference to the size of the study population	No information provided	Tim-3 and GAL-9	Flow cytometry, RT-PCR	Significantly lower Tim-3 and GAL-9 mRNA in both PBMCs and mucosal biopsies taken from patients with UC.
Xu et al.	2012	30 UC, 30 CD, 10 controls (polyps), All IBD in remission	All on Prednisolone, other treatment not documented	GAL-3	Mucosal biopsies: Immunohistochemistry, Western blots Serum: ELISA	Higher frequencies of CD98+ eosinophils were seen in the IBD intestinal mucosa than controls. Subsequent in vitro studies were done to demonstrate that flagellin can induce dendritic cells to release GAL-3, which in turn induces eosinophils to release chemical mediators via the ligation of CD98.
Block et al.	2016	22 UC, 10 controls	Mixed treatment including biologics	GAL-1, -2, -3, -4	Staining of paraffin sections from resection specimens and Immunohistochemistry	GAL-1 was not expressed on the colonic epithelium, but GAL-2-4 showed strong expression, though this diminished with increased inflammatory activity.

Papa-Gobbi et al.	2016	22 CD, 37 UC, 8 Coeliac, 9 Rejection post-transplant, 32 Controls	Not provided	GAL-1, -3, -4, -9	Mucosal biopsies: Galectin mRNA expression analysed via RT-PCR and quantitative (q) PCR	Significant increases in GAL-1 mRNA and decreases in GAL-3, -4 and -9 mRNA in severely inflamed mucosa LDA was able to discriminate between inflammation grades in IBD using GALectin expression and showed that galectin expression at remission clustered with control patients. No differential clustering was seen between CD and UC.
Cibor et al.	2019	48 UC, 77 CD, 30 Healthy controls	Mixed post treatment including biologics, 5 years median disease duration	GAL-3, GAL-3 binding protein and GAL-9	Serum: ELISAs	No significant difference between galectin levels in IBD subgroups. Serum GAL-3BP was significantly higher in active CD than controls.
Volarevic et al.	2019	65 UC, 30 controls	Mixed therapy including biologics. Median disease durations varied with severity groups, between 4.4 – 16.5 months	GAL-3	Serum: ELISA, Spectrophotometric assays Mucosal biopsies: Regulatory T cells (Tregs) isolated for flow cytometry	Higher serum GAL-3 levels in UC relative to healthy controls. However, amongst UC, GAL-3 levels were significantly higher in those with milder disease. The lowest levels seen in those with severe inflammation. Clinical severity indices significantly negatively correlated with GAL-3. GAL-3 levels strongly correlated with Kynurenine, the % of colon infiltrating Tregs and serum IL-10 levels. Finally, GAL-3 levels were also found to be higher in the stool of UC patients relative to controls.
Jovanovic et al.	2019	89 de novo UC patients	New onset disease	GAL-3	Serum and stool: ELISA Mucosal biopsies: Isolation of Tregs and flow cytometry	This study focusses on the impacts of metabolic syndrome on IBD presentation but highlights again that elevated GAL-3 levels are seen in those with milder disease and are accompanied by increased levels of IL10.
Chen et al.	2020	243 CD, UC 73, 43 Controls	Newly diagnosed	GAL-9	Publicly available transcriptomic data was mined for LSGALS9 expression Secondary analyses involving mouse models.	This paper presents a secondary analysis of publicly available data from the risk cohort (Gevers et al., 2014). Enrichment of LSGALS9 transcripts were found in inflamed mucosa, with a positive correlation with disease severity, similar in pattern to the enrichment of other genes related to T cell activation. Moderate enrichment of LSGALS9 was also found in PBMCs from this cohort.

Yu et al.	2020	97 CD, 71 UC, 40 Healthy	Mixed post treatment including biologics	GAL-1, -2, -3, -4, -7 and -8	Serum: ELISAs	Of the analysed galectins, only GAL-1 and GAL-3 were significantly different in IBD to healthy – both higher. No galectin was able to distinguish active and inactive disease.
Iwatani et al.	2020	Mixed number per sample type. Serum work included: 53 UC, 33 Controls	Details not provided	GAL-1	Serum: ELISA Mucosal biopsies: Paired inflamed / non inflamed, RT-PCR Surgical specimens: Immunohistochemistry	No significant difference in Serum GAL-1 levels between IBD and healthy. GAL-1 mRNA expression was significantly decreased in the inflamed mucosa compared to non-inflamed. There was an associated reduction in GAL-1 positive cells within the colonic lamina propria. GAL-1 tended to co-localise with CD13+ cells.
Kessel et al.	2021	40 UC, 10 CD Samples analysed after a period of patient follow up and used to predict risk of flare	Mixed post treatment including biologics	GAL-1, -7, -9	Serum: Multiple cytokines and chemokines	GAL-1 was significantly higher in those with unstable remission who went on to have a flare during the period of follow up. Though GAL-7 and -9 appear in heatmaps, the individual data is not presented.
Papa-Gobbi et al.	2021	61 UC, 36 CD, 52 controls	Mixed post treatment, specifics not provided	GAL-1	Mucosal biopsies: Isolation of lamina propria cells, Immunohistochemistry, RNA isolation for RT-PCR, western blots, binding assays	Substantial GAL-1 expression within the healthy colonic epithelium and lamina propria. In this study, significant upregulation of GAL-1 in inflamed areas on both an mRNA and protein level. GAL-1 expression increased in controls after stimulation ex vivo with TNF. GAL-1 was able initiate apoptosis of lamina propria CD3+ T cells (from both control and non-inflamed IBD mucosa). GAL-1 binding to CD4+ and CD8+ T cells was decreased in T cells isolated from inflamed areas.
Pesole et al.	2023	30 CD, 18 UC	All patients starting infliximab at enrolment	GAL-3 binding protein	Serum: ELISAs	GAL-3BP correlated with CRP but no other parameters. Patients who went on to develop antibodies to infliximab had significantly higher GAL-3BP at baseline in CD, but not reaching significance for UC. Non responders to Infliximab had higher GAL3-BP at baseline.

1.5.3 Conclusion

The galectins represent a promising area for further study within IBD. Existing studies are limited and include disparate cohorts in terms of disease state and treatment. Despite this, several promising findings relating to disease severity and outcomes, in particular relating to GAL-1, -3 and -9 are present in the literature. Clear differences have been demonstrated between IBD and health, in addition to those between inflamed and non-inflamed mucosa. The differences seen regarding disease severity and treatment outcome would best be measured at disease onset to exclude any influence that treatments are liable to have on galectin levels. Further study of galectin levels, for instance in serum, amongst pre-treatment cohorts may help predict disease course and enable the delivery of more personalised medicine.

Through prospective evaluation integrating multi-omics data from pre-treatment patients, with a particular focus on interactions with the microbiome, we hope to further understand the role that galectins play in IBD onset and can play in helping to personalise our management of IBD.

1.6 Thesis Hypothesis and aims:

The overall hypothesis explored hereafter is twofold. The first, relating to our clinical model, is that by delivering a tailored diagnostic pathway we can shorten the time to diagnosis and treatment for those with IBD. Alongside this, through better utilisation clinical tools to stratify patients we can further personalise the treatment plans offer to consequently improve patient outcomes.

The second hypothesis is that GAL-1, -3 and -9 are significant mediators of host-immune responses at IBD onset that can be linked to gut microbiome perturbations, used to guide diagnosis and predict likelihood of treatment response early in the disease course.

To fully explore the two hypotheses, the thesis has the following aims:

1. Design and implement a bespoke diagnostic pathway dedicated to patients with suspected inflammatory bowel disease.
    a. The cohort will be characterised in depth from a clinical standpoint to seek key signals that can speed diagnosis and focus clinical prioritisation.

2. Characterise the host immune and microbial pathways associated with the onset of IBD.

a. Quantify the expression of specific galectins in human serum from patients with newly diagnosed inflammatory bowel disease, prior to the initiation of treatment.
b. Evaluate the faecal microbiome in this same cohort, relative to patients without IBD.
    i. Utilise 16S rRNA sequencing for broad differences and shotgun metagenomics deeper characterisation within patients with IBD.
c. Integrate our microbiome data with publicly available raw datasets to minimise methodological interference and assess for core themes across patient groups and sample types

3. Integrate the galectin and microbiome data to identify novel interactions and relationships underpinning IBD onset and develop predictive markers that can help identify aggressive disease phenotypes and behaviours at onset.

CHAPTER 2: Materials and Methods

2.1 Integration of a ‘Rapid access’ clinical pathway with research

2.1.1 Defining the patient cohorts

The clinical pathway *(fully explored in Chapter 3)* focusses on the diagnosis and treatment of new referrals where IBD was suspected. It was established on the backdrop of spiralling waiting times and as such had to be pragmatic regarding patient selection, ensuring all urgent cases were seen promptly. In short, the inclusion criteria were not applied strictly. In general, patients did:

- have symptoms that the triaging clinician felt were compatible with an IBD diagnosis.
- unless clinical suspicion very strong, have supporting evidence in the form of ‘elevated’ faecal calprotectin (FCP). No set cut-off applied and patients with only one result at referral were still seen.

A repeat FCP sample kit was sent to all patients in advance of their appointment, but failure to return this did not prevent progression through the clinical pathway. Patients referred with symptoms suspicious for IBD but meeting ‘straight to test’ colorectal cancer referral criteria were managed using existing pathways. However, the clinic also provided a single point of access for patients whereby IBD was suspected after their ‘two-week wait’ colonoscopy. Where possible, inpatients presenting acutely with suspected IBD were also integrated with the pathway, either providing a path to early outpatient colonoscopy in those well enough or supporting ongoing care on discharge. All final IBD diagnoses allocated to patients during clinical follow up were made in line with the British Society of Gastroenterology

(Lamb et al., 2021) and European Crohn's and Colitis Organisation (Maaser et al., 2019) guidelines.

Clearly, for the research component of the pathway, inclusion criteria were stricter and applied rigidly. These were:

- Age ≥ 18 years old
- Suspected or newly diagnosed IBD
- Prior to the initiation of any conventional pharmacological treatment for IBD
- Willing and able to provide informed consent

Exclusion criteria:

- Age <18 years old
- Initially, a positive lateral flow test for COVID-19 excluded participants, though lateral flow testing was no longer mandated 12 months into the project.
- Patients who have previously received treatment for IBD or other immune mediated gastrointestinal diseases
- Unable to give informed consent
- Pregnancy

All referrals were initially screened by members of the gastroenterology clinical team. Where IBD was suspected, these referrals were passed on to the inception clinic team. Enhanced triage was undertaken at this point, both to allow prioritisation based on clinical need and assess eligibility based on clinical details. As part of the

appointment booking process, patients were approached by the administrative team to obtain consent for further contact regarding research. Those consenting to this were sent a patient information sheet (email or post) regarding the project, to be received at least 72 hours prior to the appointment. Inpatients agreeing to contact about research were given 24 hours to consider the patient information sheet prior to consent. For those who were post colonoscopy and had a clear need for urgent treatment initiation remotely (via same day telephone review), the research component was not discussed, and they were brought in for a purely clinical review subsequently.

For outpatients, consent was obtained either during the first outpatient consultation, or rarely, on the day of colonoscopy. For inpatients, consent was taken the day after patient receipt of the patient information sheet. In these cases, patients were only recruited if medical treatment had not been initiated at the discretion of the medical team (for example whilst awaiting stool cultures to exclude infection). The flow through the research arm of the pathway is summarised in ***Figure 2 – 1***.

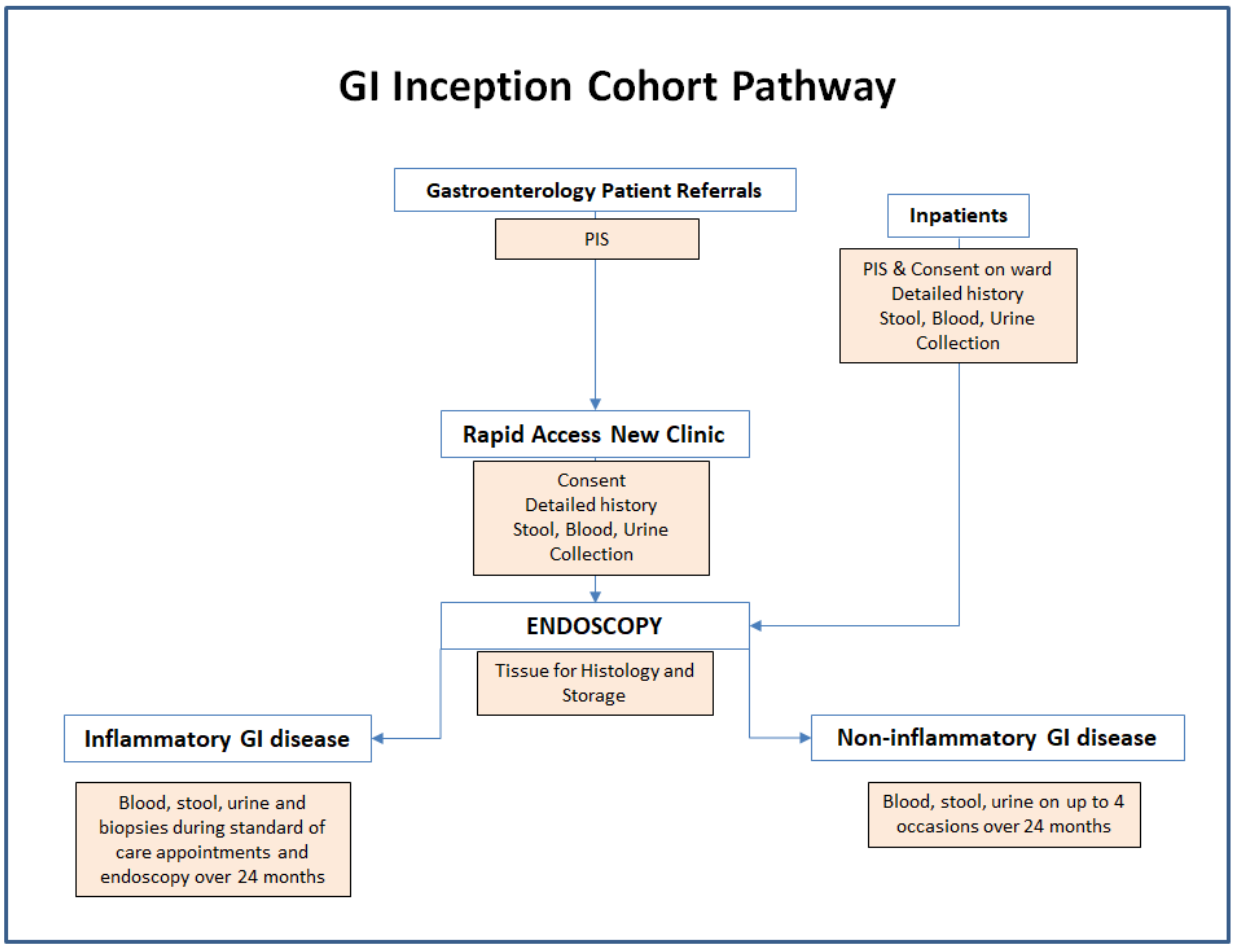


Figure 2 - 1: Integrated clinical and research pathway for patients with suspected IBD

This summarises patient flow and sample acquisition through the diagnosis pathway. Urine samples were ultimately not obtained. The Non-Inflammatory GI disease cohort formed a control group for different components of our analyses. However, it also represents a highly heterogenous group ranging from irritable bowel syndrome (IBS) to colorectal cancer. Filtering of the cohort and separate acquisition of true healthy controls was necessary for some comparisons. PIS = Patient Information Sheet.

2.1.2 Ethics approval for pathway

From January to the end of June 2021, acquisition of blood for serum (for galectin analyses) and stool samples for microbial profiling in patients was given by the Yorkshire & The Humber – Bradford Leeds Research Ethics Committee (REC reference: 16/YH/0100). From July 2021, a new protocol was approved covering the continued acquisition of these samples in addition to gut mucosal biopsies for host transcriptomics. This approval was received from the London – Bloomsbury Research Ethics Committee (REC reference 21/PR/0515).

2.1.3 Sample collection protocols for serum

Serum samples were typically obtained prior to diagnosis at the first 'new patient' appointment. A minority were collected on the day of colonoscopy. All samples were obtained prior to treatment. As standard, 12ml of blood was collected in clot activator and separation gel tubes. After being allowed to clot for 15-30 mins, samples were transferred to the laboratory. Here, they were centrifuged at ~1000g for 15 minutes. Separated serum was then carefully transferred into a fresh tube, mixed by inversion and divided in 1ml aliquots into cryovials. Samples were frozen at -80°C within 1.5 hours of collection. Samples remained at -80°C until further aliquoting was required in anticipation of assays undertaken. Typically, all samples had two freeze thaw cycles prior to analysis.

2.1.4 Sample collection protocols for faecal samples

Patients screened as eligible for the research, and consenting to be contacted, were sent a sample collection tube for a faecal sample and instructed to bring this along to their clinic appointment. Patients were also sent a repeat sample collection tube for a

faecal calprotectin and where necessary, samples for faecal microscopy, culture, sensitivity and clostridium difficile testing.

Depending on stool frequency, patients were instructed to produce samples the evening before or the morning of the clinic appointment. To ensure microbial stability of the research sample, samples were collected utilising the DNA Genotek® OMNIgene GUT OM-200® stool collection kits (DNA Genotek, 2022; Macklaim et al., 2022). Samples were obtained as per the manufacturer's protocol, an adapted version of which is presented in ***Table 2 – 1***.

Table 2 - 1: Abridged version of the DNA Genotek OMNIgene GUT OM-200 sample collection protocol (Adapted from DNA Genotek, 2022).

1.	Empty your bladder before attempting to collect the faecal sample. When collecting, please ensure it is collected free of urine or toilet water.
2.	Remove the purple cap to expose the lower yellow cap. Set the purple cap aside for later use. Do not spill or remove the liquid within the sample tube.
3.	Use the spatula to collect a small amount of faecal material and transfer this into the yellow cap top. A spoon is also available for liquid faecal samples.
4.	Scrape horizontally across the yellow cap top to level the sample and remove excess
5.	Replace the purple cap on top of the yellow cap and screw tightly until closed.
6.	The sample pot contains a buffer and a ball bearing to allow adequate mixing. Please shake the sealed tube as hard and as fast as possible in a back-and-forth motion for 30 seconds.
7.	Even with the ball bearing, it is not expected that all particles will dissolve but please continue mixing beyond 30 seconds if large particles remain.
8.	Keep the sample between 15 - 25°C until handed to the research team.

Product literature for these samples' states stability at room temperature for 60 days, and for up to 1 year at -20°C or -80°C if stored in the original tube (longer if aliquoted into cryovials). Samples were kept at room temperature prior to handing over to the research team. If DNA extraction was possible within 1 week then samples were kept at room temperature, otherwise were transferred to a -80°C freezer in the original tube until DNA extraction was possible, with only one freeze thaw cycle prior to extraction.

Though desirable, dedicated samples for faecal metabolomic profiling were not obtained. This, in part, owed to the burden of faecal samples patients were already providing that were integral to clinical diagnosis and management, and the prioritisation of microbiome profiling in this context. Furthermore, the need for fresh samples for accurate metabolomic analysis was not something that could be consistently achieved. The use of Genotek kits overcame this hurdle with regard to microbiome analyses, but these kits are not validated for the subsequent performance of metabolomic analyses.

Where patients were recruited after the diagnostic colonoscopy had already taken place, faecal samples for microbiome analysis were not obtained unless at least 14 days after bowel preparation, due to the well-established impact of bowel cleansing solutions on the gut microbiome (Nagata et al., 2019). Whilst mucosal biopsies were also obtained during this research pathway, this data is not included in this thesis and thus the methodology is not included.

2.1.5 Statistical analyses and modelling within the clinical dataset

Performance indices relating to time to review, symptom duration and time to diagnosis were collected from all patients progressing through the pathway at the Queen Elizabeth Hospital, with more sporadic data collection in Heartlands. All patients seen via the pathway had a standardised 13-point symptom history taken at the index appointment, prior to a diagnosis being established. A subgroup of patients also completed an IBD disk score and a Hospital Anxiety and Depression scale (HADS). Responses were recorded electronically. Diagnoses were established using history, biochemistry, endoscopy, histological and radiological criteria in line with The European Crohn's and Colitis Organisation guidelines (Maaser et al., 2018). Detailed clinical indices were collected prospectively, alongside outcome data. The clinical indices collected are explored further in section 2.5. prospectively.

Analyses based on this data were undertaken utilising Jamovi (Jamovi, 2022), R packages (R Core Team, 2021) and JASP (JASP team, 2024). Regression modelling was undertaken in Jamovi using additional packages (Lenth, 2020, Ripley and Venables, 2016, Thiele, 2019 and Friesen et al., 2019). Analyses included only those with an established final diagnosis. All key datasets presented followed a skewed distribution (1st FCP Shapiro-Wilk [SW] 0.846 $p<0.001$, 2nd FCP SW 0.697 $p<0.001$, difference between FCPs SW 0.926 $p<0.002$, symptom duration SW 0.589 $p<0.001$, age SW 0.95 $p<0.001$, BMI SW 0.94 $p<0.001$, IBD disk score SW 0.98 $p=0.006$). As such, non-parametric tests were utilised throughout. Mann-Whitney U tests were used for two groups, with Kruskal -Wallis for more than two groups (and Dunn test for pairwise comparison, p values use holm correction to account for population wide differences). Chi-squared tests (global and pairwise) were used to demonstrate

significant differences in the proportion of categorical variables. For repeated measures, a non-parametric Wilcoxon-rank is presented.

The predictive models utilised a complete-case analysis approach. The sample size was pragmatic and dictated by patient availability. Two models were developed. The two major models presented focussed on symptom histories, initially in isolation and then with the addition of FCP results. The first multinomial logistic regression used symptoms and predicted either CD, UC or non-IBD diagnosis. Odds ratios predicting both CD and UC over a reference diagnosis of 'non-IBD' were calculated. All parameters presented were modelled as factors with binary 'Yes'/'No' responses ('No' given as the reference level for all) bar 'Blood type' ('Mixed', 'Anorectal' or 'None' with 'None' as reference) and 'Smoking Status' ('Current', 'Ex', 'Non' with 'Non' as reference). The second model was a binomial logistic regression predicting 'IBD' vs 'Non IBD'. The same methodology was applied to the symptom profiles. Two approaches were taken to integrating FCP results. In the main model, 1st and 2nd FCPs were added as covariates. To assess the performance of FCP cut offs alongside specific symptoms, an alternative approach was used. Overall FCP levels were removed as covariates. A binary 'Yes'/'No' response to achieving a single FCP cut off was used instead and then added as a factor. The quality of fit for models is presented using McFadden's PseudoR^2 and the overall model test. Other small models follow the same basic principles with additional detail given where they are utilised in the text.

2.2 Galectin Profiling

2.2.1 ELISA Protocols

The main point of focus has been to profile GAL-1, GAL-3 and GAL-9 at the onset of Inflammatory bowel disease. To do this, Enzyme-linked Immunosorbent Assays (ELISA) have been undertaken. In addition to the galectins above, associated cytokines (Tumour Necrosis Factor alpha [TNFα], Interleukin-6, -12 and -23) have been quantified in serum using further ELISAs. Finally, the first run of galectin profiling included GAL-10 and Tim-3, though this was not pursued throughout.

For each ELISA, stored serum was thawed on ice and vortexed during this process and again just prior to aliquoting. Serum was pre-aliquoted into 96-well plates for dilution as per the manufacturer's instructions for a given ELISA ***(Table 2 -2).*** This was done for all planned ELISAs per run so that these plates could be re-frozen and only thawed when required, to avoid repeated-freeze thaw. Standard protocols for each ELISA were followed. Samples were processed in three time points over the course of the project with selected samples repeated in each run to ensure reasonably consistency (within the context of an extra freeze thaw cycle) between each run.

Table 2 - 2: Summary of ELISA kits utilised for galectin profiling and comparative inflammatory cytokines

	ELISA type	Manufacturer	Dilution used	Standard range
GAL-1	Quantikine	R&D	1 in 4	15.6-1000pg/ml
GAL-3			1 in 2	
GAL-9			1 in 4	
GAL-10	Standard	Novus Bio	1 in 10	
Tim-3	Duoset	R&D	1 in 10	62.5-4000pg/ml
TNF	Quantikine		None	15.6-1000pg/ml
IL6			None	31.2-2000pg/ml
IL23			None	39-2500pg/ml

R&D = R&D systems, a Bio-Techne brand.

Standard curves and samples were run in duplicate wherever possible. Optical density was determined by reading the plates at different wavelengths. Readings were taken at 450 and 540nm using a Biotek Synergy HT microplate reader and Kc4 analysis software within 30 minutes of stopping the ELISA. The coefficient of variation (CV) between each duplicate was calculated for the 450nm reading and again for the delta value to ensure data was consistent. The delta values were then averaged, and the average of the blank value from the standard curve subtracted.

2.2.2 Statistical analyses

Concentration data for the samples was then interpolated from the standard curve using a four-parameter logistic curve fit in Prism (GraphPad, California, US). Values above the upper limit of the standard curve were obtained using a simple linear regression. Results were corrected for initial dilution to generate the data used in the analyses.

Interpolated values were analysed in greater detail using JASP (JASP team, 2024), Jamovi (Jamovi, 2022) and R (R Core Team, 2021), with additional modules from several sources (Wickham et al., 2018; Lenth, 2020; Patil, 2018; Singmann, 2018; Fox and Weisberg, 2020). A power calculation was not utilised to decide on cohort size given this is exploratory work in a pre-treatment cohort where there are limited opportunities to recruit (given the need to approach, recruit and obtain samples prior to treatment initiation, without delaying therapy) and a paucity of existing datasets. All baseline galectin measurements followed a skewed distribution. This was true of both the pilot (GAL-1 Shapiro-Wilk [SW] 0.62 $p<.001$, GAL-3 SW 0.68 $p<0.001$, GAL-9 SW 0.45 $p<.001$) and the inception datasets (GAL-1 SW 0.65 $p<.001$, GAL-3 SW 0.87 $p<.001$, GAL-9 SW 0.91 $p<0.001$). Consequently, non-parametric testing was used. When comparing two independent data sets, a Mann Whitney U was utilised with a standard p-value threshold of 0.05. When comparing two paired groups, a Wilcoxon-signed-rank was utilised. For tests including more than two independent groups, a one-way ANOVA (Kruskal-Wallis test by ranks) was used, and p values are presented with correction using the Holm method to control for the family wise error rate (Holm, 1979). Where testing is undertaken using more than two groups of paired samples over multiple time points, a non-parametric repeated measures ANOVA (Friedman test) was utilised. Again, p-values are presented after correction using the Holm method. Microbiome analytic methods are addressed in more detail subsequently. At times, where subgroup analyses are desired in an overall population with more than two groups, a Wilcoxon-signed-rang was again utilised.

Approaches to analysis relating to treatment response are explored further in ***Section 2.5.***

2.3 Microbiome analyses

Evaluation of the baseline gut microbiome has been undertaken on faecal samples only. 16S rRNA sequencing and shotgun metagenomics have been undertaken though the initial DNA extraction following the same pathway for both.

2.3.1 Microbial DNA extraction from human faecal material

Initial sample handling and storage has been discussed. In retrospect, samples should have been aliquoted into individual cryovials to prevent repeated freeze-thaw for the overall sample whilst obtaining adequate microbial DNA concentrations. The vast majority only required a single extraction for adequate concentration and no sample was exposed to >4 cycles (OMNIgene product literature states stable for up to 6 cycles). However, this process limited potential to use the samples for any associated analyses.

Microbial DNA extraction was performed utilising the Qiagen QIAamp Fast DNA Stool minikit (Qiagen, Hilden Germany). The standard protocol was modified to increase yield. These changes were made based on prior experience and optimisation undertaken by predecessors with our lab. The modifications are detailed below, with the remaining elements of completed as per the standard product protocol:

1. Thermal lysis temperature increased to 95°C as standard (recommended temperature for cells that are difficult to lyse)
2. Following thermal lysis, a mechanical lysis step was added. 300mg of glass beads (0.1mm, Qiagen) were added to the homogenised inhibitEX / sample

solution after thermal lysis. The sample was then placed in Qiagen Tissuelyser 2 and subjected to 1 minute of shaking at 30Hz (1800 oscillations per minute). This was followed by 30 seconds of rest and the process repeated on 3 occasions.

3. Centrifugation time to pellet the stool particles after this point was increased to 5 minutes

Extracted microbial DNA was quantified via fluorescence utilising a Qubit Fluorometer and, at this stage, the Invitrogen dsDNA Broad Range assay kit (ThermoFisher Scientific, Cat. No Q32853). A value of more than 1ng/ul was considered acceptable post extract for the sample to be taken forwards for polymerase chain reaction (PCR). In total microbial DNA was extracted from 245 samples. Levels in excess of the target DNA were achieved on the first attempt (x1 freeze/thaw) in 208 (85%) samples. 22 (9%) had x2 freeze/thaws, 3 (1%) had x3 and 2 (1%) had x4. There were 10 (4%) samples where quality prevented the extraction of any measurable DNA over multiple attempts.

2.3.2 16S rRNA sequencing

Sequencing was undertaken with minor deviations from version 1 of the 16S Illumina Amplicon Protocol (Earth Microbiome Project, 2018), with modifications for MiSeq as per Caporaso et al. (2012). Primers 515F-806R targeting the V4 region of the 16SS rRNA were utilised. Forward (515F) primers contained the unique Golay barcodes. From the initial methods described by Caporaso et al. (2012), degeneracy has been added to forward and reverse primers to remove recognised biases against particular prokaryotes. Forward primers were ordered salted from Integrated DNA

technologies and resuspended in nuclease free water prior to further dilution for sequencing. Reverse primers were ordered from Sigma Aldrich with required volume aliquoted separately and diluted per PCR. This methodology had an expected amplicon size of 390bp.

Given the longitudinal sample collection throughout the project, samples underwent PCR in two batches at separate time points, though utilising the same protocol for each. The first PCR batch involved 49 samples, whilst the second 148 samples. Negative controls were run with each batch.

2.3.2.1 PCR Protocol

Extracted template DNA was normalised to 1ng/ul, with 3ul submitted per reaction to make the final reaction volumes described in ***Figure 2-2***. The PCR protocol utilised had been previously validated internally and was provided by a member of my supervisory team (Quraishi, 2020). PCR reactions were undertaken utilising a BIO-RAD C1000 Touch Thermal Cycler.

Reagent (x1)	Volume
PCR-grade water	25.0 µL
PCR master mix (2x)	20.0 µL
Forward primer (5 µM)	1 µL
Reverse primer (5 µM)	1 µL
Template DNA (1 ng/ul)	3.0 µL
Total reaction volume	50.0 µL

Temp	Time, 96-well
94 °C	3 min
94 °C	45 s
50 °C	60 s
72 °C	90 s
72 °C	10 min
4 °C	hold

Steps repeated for x33 cycles total

Figure 2 - 2: PCR reaction mixture and thermal cycler conditions

Each 50ul reaction volume was run as one well within a 96-well plate, rather than in duplicate or triplicate. Invitrogen Platinum Green Hot Start PCR Master Mix 2x (ThermoFisher Scientific, Cat no 13001013) was utilised with the provided PCR-grade water used in the reactions.

2.3.2.2 Library clean up

Following PCR, library preparation and clean up was undertaken using Agencourt AMPure XP bead-based reagent (Beckman Coulter, Product no A63882). Within a 96-well plate, each individual sample's 50ul reaction volume was combined with 80ul of bead solution and mixed with a pipette x10. After 5 minutes of incubation at room temperature, the library was placed on a magnetic rack. After the liquid had cleared (allowing 2-4minutes for this), liquid was removed with a pipette and discarded. The bead pellet was washed with 200ul of freshly prepared 80% Ethanol. After resting for 30 seconds, the liquid was removed and the process then repeated. After the step had been repeated, careful checking to ensure all remaining liquid had been removed was undertaken and an additional 2 minutes of air drying undertaken to ensure no residual ethanol remained. The plate was then removed from the magnetic rack and the bead pellet was resuspended in 23ul of nuclease free water. To achieve resuspension, mixing with a pipette was undertaken on at least 10 occasions. The 96 well plate was then placed back on to the magnetic rack. Once the liquid had again cleared, 20ul was removed into a new 96 well plate and this was applied to each sample.

2.3.2.3 Normalisation

To normalise the samples, post reaction microbial DNA concentrations were again analysed by Fluorescence, again via a Qubit Fluorometer. For this, the Invitrogen Qubit dsDNA HS Assay kit (ThermoFisher Scientific, Q32854) was used to quantify individual DNA concentrations. A D1000 TapeStation was used to check amplicon sizes in 10% of samples and the subsequent sample pool prior to sequencing.

TapeStation outputs for each run are displayed in ***Figure 2-3***. It is notable that individual samples were not analysed by TapeStation on the first attempt to sequence the second sample batch. Only when samples were pooled and analysed via TapeStation was it identified the incorrect Universal Reverse Primer had been utilised, generating an Amplicon size of 1080bp. Whilst frustrating, this did demonstrate that effective safeguards were present within the methodology and mistakes were identified prior to sequencing. This sample pool was discarded, and PCR process repeated with the appropriate reverse primer. The methodology was further adjusted to identify mistakes earlier, with both individual samples and the pool analysed by TapeStation on the rerun. Normalisation was undertaken based on the post PCR microbial DNA concentration, normalising all samples to the lowest concentration achieved. For the first submission, samples were normalised to 7ng/ul with 8ul per sample pooled. Due to improved performance across the second PCR, samples were normalised to 20ng/ul with 5ul per sample added to the pool for submission.

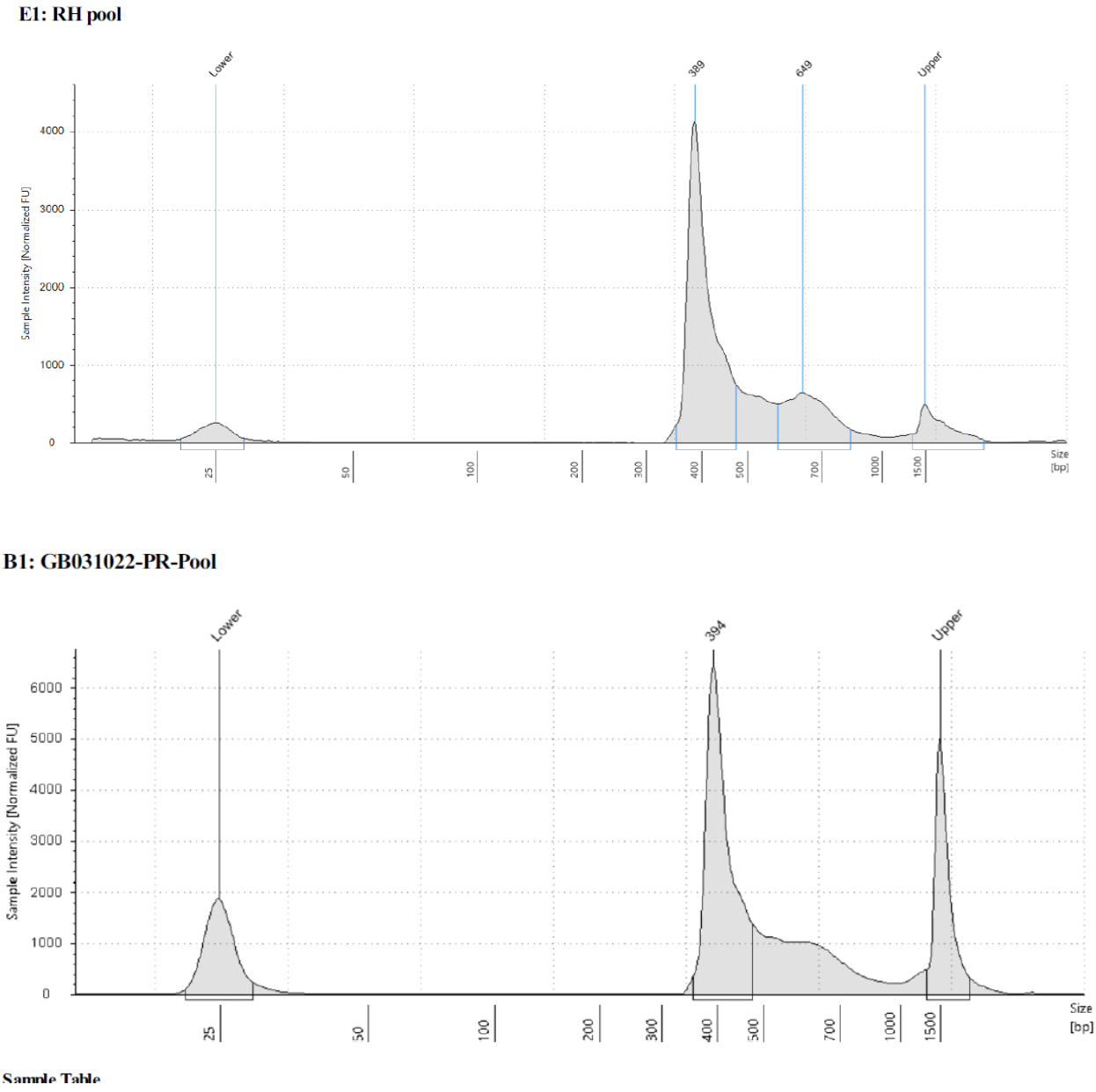


Figure 2 - 3: TapeStation Analyses of pooled 16S rRNA libraries from both PCR runs

For the first run, the average amplicon size was 389 base pairs, whilst the second was 394 base pairs.

2.3.2.4 Sequencing protocol

The prepared pooled library was submitted to Genomics Birmingham, based within the University of Birmingham, who then undertook the sequencing procedure. For the first sequencing run, the library was denatured with a 0.2 N NaOH solution and diluted with a HT1 buffer to obtain a 8pM library concentration that could then be loaded for sequencing. Prior to this, Read 1 and Read 2 sequencing primers were spiked in alongside an Index Sequence primer. Additionally, freshly prepared denatured PhiX was added to the library in volumes commensurate with a 20% spike-in. Paired end sequencing was performed using the Illumina MiSeq sequencing platform (Illumina, San Diego), specifically via MS-102-2002 MiSeqv2 300 flow cell using a 2x 150bp cycle programme, aiming for 50,000 reads/library. A Q30 score of 80% or greater was desirable to ensure accuracy. For the second sample set, lower sample diversity within the 16S library resulted in repeated over clustering despite using a 20% PhiX spike in and a 40% reduction in the library concentration. To overcome this, the PhiX spike in was increased to 30% and the library diluted further to 3pM. Ultimately, the first run achieved a Q30 score of 80.25% and the second 79.57%.

2.3.3 Bioinformatic workflows for 16S rRNA profiling of the faecal Microbiome at IBD onset

Sequencing between the two runs generated 8.39gbp of read data. These outputs were transferred to Illumina BaseSpace and downloaded accordingly. Demultiplexed FASTQ files were processed using the Quantitative Insights Into Microbial Ecology 2 (QIIME) workflow (Bolyen et al., 2019). Within QIIME, denoising and quality control was undertaken using the Divisive Amplicon Denoising Algorithm 2 (DADA2) pipeline

(Callahan, 2016). Forward and reverse read counts were visualised alongside mean quality score. Trimming at this stage was undertaken based on maintaining quality scores above Q30. Individual read counts were visualised per sample trimmed above the counts generated by negative controls. Utilisation of the Earth Microbiome Project and associated custom primers should prevent primers being found in sequences but utilising DADA2 will further correct amplicon data and filter reads associated with an PhiX spiked in prior to the sequencing process. This process produces de-noised amplicon sequence variants (ASVs) which are utilised to form a higher-resolution version of the traditional OTU table. Identification of chimeras is then easier due to the increased accuracy associated with ASVs. Thereafter, taxonomy can be assigned to generate a phylogenetic tree. At this stage, QIIME2's own MAFFT (Multiple Alignment Fast Fourier Transform) multiple sequence alignment programme can be utilised to conduct diversity analyses and produce counts tables.

With our data, reads were trimmed above the negative control values and counts tables generated. Alpha and Beta Diversity analyses were performed directly with QIIME2 diversity plug in. The counts table was then converted to a dataframe within R and associated metadata uploaded to perform further analyses. In line with the studies presented in our earlier systematic review, offered alpha diversity indices include Shannon Entropy, Simpson index, Faith's phylogenetic diversity and the Chao1 index. Beta diversity is shown as Bray-Curtis dissimilarity and Unifrac distance.

For comparisons between patient groups regarding microbial abundance, MaAslin2 was predominantly utilised. This is an R package able to determine multivariable associations between microbial meta'omic features utilising general linear models to generate correlation coefficients which can be used to confer Log2 fold change, referred to subsequently as L2FC (Mallick et al., 2021). Across all these analyses, all values considered significant in the text have a p value of <0.05. All data is then corrected for multiple testing. Generated q-values, or false discovery rates, of <0.05 are considered 'strong associations' marked with '***'. This is in line with the authoritative IBD inception paper from Schirmer et al. (2018) and Morgan et al. (2012), correlations with an FDR of <0.2 will also be presented as a 'weak association' without additional marking. Summary plots of fold changes were then generated using ggstatsplot2 in R (Patil, 2018). For specific questions, correction for baseline inflammation (based upon FCP) was undertaken. Patients enrolled in the research may have one or two FCP results available. The second result is taken at the same time as the sample for sequencing and as such is more closely related to the levels of mucosal inflammation at the time of sampling (and therefore used for the microbial associations with FCP). However, in the 16S dataset, only 100 had a second result, whilst this was 55 for the metagenomic data. This would exclude those with missing data from the planned analysis. As such, a composite FCP is used for correction of other variables, where the first FCP result is used for those missing a second sample. This method accepts slightly lower accuracy to include the maximum number of samples, whilst also acknowledging the strong correlation between first and second FCP result seen amongst all IBD patients being diagnosed via our inception pathway (n=187, Spearman's Rho 0.476 p<0.001). The same is

true to analyses relating to FCP, where the composite value is presented unless otherwise stated.

In addition to this approach, two other pipelines were utilised during the 16S analyses. With the overall 16S data Topic modelling was utilised. This utilises a form of natural language processing, Latent Dirichlet Allocation, to classify ‘documents’ and identify natural unobserved groupings or ‘topics’. In the context of the microbiome, ‘document’ would be the biological sample (in this case the faecal microbiome), ‘term’ a single feature e.g. taxa and ‘topic’ a community type within the microbiome. (Sankaran and Holmes, 2019). In this form of analysis, documents can have fractional membership across a set of topics, preventing a binary and limited assignment per term and ideal where a multitude of complex relationships exist. This form of modelling is probabilistic and may identify themes that would otherwise remain hidden using traditional testing methodologies. Finally, when trying to integrate galectin and microbial datasets, Phyloseq (McMurdie and Holmes, 2013) has been utilised, with pheatmap for visualisations (Kolde, 2019). This allows relative correlations to be presented with adjusted p values considering population wide differences across all variables selected. It readily allows the construction of large visualisations that account for other confounding factors such as differences in patient demographics.

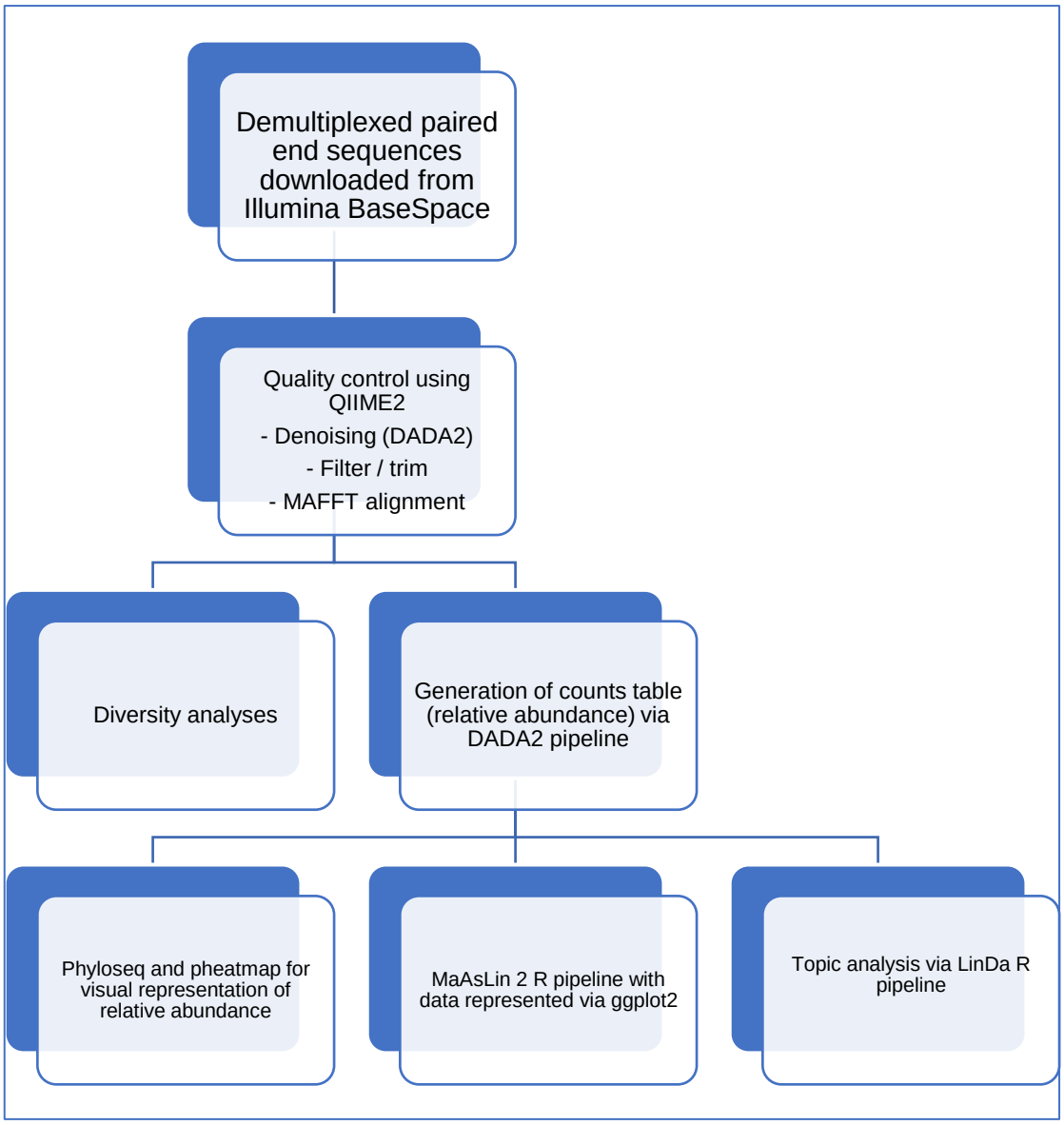


Figure 2 - 4: Summary of workflows and applied analytic pipelines utilised for the 16S rRNA dataset.

By utilising both traditional analytical methods and additional probabilistic modalities, both apparent and more obscure themes within the dataset will be sought. I have delivered QIIME, MaAsLin and Phyloseq analyses in an independent manner but received bioinformatic support with the Topic analysis. MAFFT = Multiple alignment using fast Fourier transform, DADA2 = Divisive Amplicon Denoising Algorithm 2.

2.3.4 Defining the cohorts.

Early analyses of our dataset (Rimmer et al., 2022) included a historic cohort of healthy controls shared with permissions from Dr Amanda Rossiter. However, access to further healthy individuals to provide faeces samples was not possible. In line with existing inception IBD literature, and to fill the identified gap for faecal samples in the published literature, symptomatic controls in whom IBD was excluded at colonoscopy were used as a comparator. . Initially, all obtained samples were sequenced for consideration of utilisation as controls after diagnostic confirmation. However, several patients were diagnosed with highly relevant organic pathologies with marked intestinal mucosal abnormalities other than IBD. As such, these individuals were excluded. Elaboration regarding samples excluded is given in the results section of Chapter 5.

2.4 Shotgun Metagenomics for analysis of the gut microbiome

2.4.1 Rationale

Whilst 16S rRNA sequencing is a reliable method of bacterial taxonomic estimation, the choice of primer utilised can lead to bias in the representation of taxonomic units and does not allow the depth of analysis associated with metagenomic evaluation (Laudadio et al., 2018; Durazzi et al., 2021). When adequate sequencing depths are chosen, the identification of less abundant taxa which are biologically meaningful but otherwise missed is possible.

Whilst the functional profile of a microbial community can be inferred from 16S datasets using modalities such as Phylogenetic Investigation of Communities by

Reconstruction of Unobserved States (PICRUSt), this has limitations. In its latest iteration (PICRUSt2), predictions are inclined towards existing reference genomes. The lower resolution of sequencing afforded by 16S approaches does not allow functionality to be established at a strain-specific level (Douglas et al., 2020). By utilising Shotgun Metagenomics, long DNA molecules such as complete chromosomes are fragmented and sequenced (Weinstock, 2012). This allows functional genes within the sample to also be analysed and functional contributions to be delineated.

With the benefit of these additional outputs in mind, samples from 85 IBD patients were submitted for analysis without a control cohort. The aim of this was to evaluate different functional contributions in patients presenting with vastly different IBD phenotypes and disease courses required different levels of medical intervention.

It is recognised that to achieve full characterisation of low abundance taxa (typically <1%) it is necessary to undertake deep metagenomic sequencing. This however is associated with cost implications. Available comparisons would suggest that shallow sequencing depths (500,000 reads) correspond with ultra-deep (>2.5 billion reads) sequencing for up to 97% of species composition and 99% of metagenomic profiles (Hillman et al., 2018). Set again the financial limitations of our project, a sequencing depth of 2 million reads was selected. This was hoped to provide an adequate depth to provide added information from 16S, particularly regarding functional profiles, whilst also enabling a broader selection of our IBD patients with matched ELISA and single cell sequencing data to be included.

2.4.2 Shotgun Metagenomics library preparation

Microbial DNA was extracted using the same methodology as was applied for 16S rRNA sequencing. 85 baseline samples were submitted which included and several of the banked samples previously submitted for 16S. From this point, the library preparation and sequencing were undertaken by the team within Genomics Birmingham in the University.

Samples were re-quality checked by the Quant-IT DNA assay (ThermoFisher), utilising a Fluostar plate reader (BMG Labtech). Libraries were prepared using NEBNext Ultra II FS DNA Library Prep Kit for Illumina and NEBNext Multiplex Oligos for Illumina (New England Biolabs, Massachusetts. Cat no E7645L and E7335L). The Library Prep kit has a working material range of 1pg – 1ug and to normalise for the lowest submitted concentration, 20ng of template DNA was inputted per sample. Submitted DNA was fragmented enzymatically for 20 minutes to achieve 150-350bp size. Library preparation was undertaken in line with the manufacturer's instructions. After PCR (6 cycles), concentrations were rechecked again using the Quant-IT DNA assay. 16 of the 85 submitted were also checked using the high sensitivity DNA Qubit assay (ThermoFisher) and high sensitivity D1000 Screentape on the Agilent Tapestation to check amplicon size. The library was pool in equimolar amounts and again quantified using the HS DNA qubit assay and HS D1000 screentape.

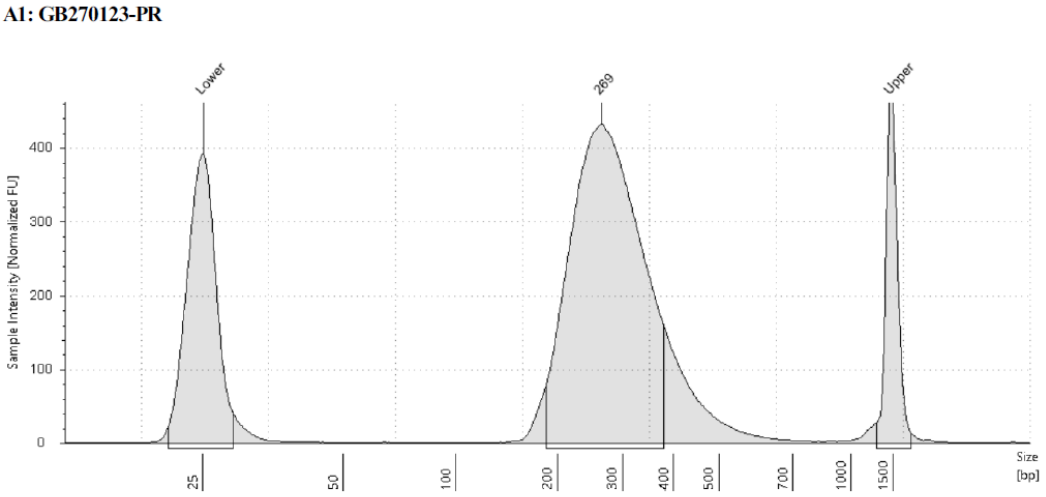


Figure 2 - 5: TapeStation readout for pooled DNA submitted for Shotgun Metagenomics

Using the DNA Qubit hsDNA assay and D1000 screentape, the pool yielded a concentration of 2.2ng/ul, amplicon size of 269bp and molar concentration of 13 nM.

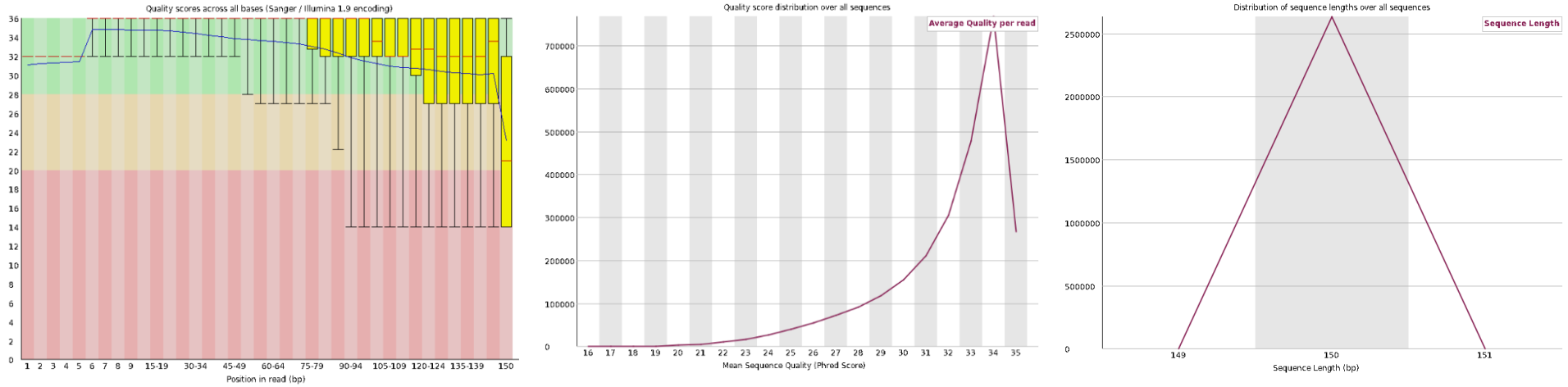
The pool was taken in to sequencing at 4nM with a 1% PhiX spike in. Sequencing was undertaken using a NextSeq550 system (Illumina) and MID 300 cycle (150 paired end) 150/8/8/150 flow cell. This yielded 62.15 Gbp, with an average %Q30 of 80.82. No host depletion was undertaken during sequencing, given the typically high bacterial load in faecal samples and in line with other inception IBD publications present this type of date (Gevers et al., 2014, Lloyd Price et al., 2021.).

2.4.3 Shotgun data processing and analytic methods

Data processing and quality control was undertaken using the HUMAnN 3.0 workflow (Beghini et al., 2021). A median of 4.3 million reads were obtained per sample. This fell to 3.9 million after trimming and 3.6 million after decontamination.

Abundance data was mapped against the ChocoPhlAn database, whilst functional pathway readouts were derived from the MetaCyc database. As an exemplar of the quality scores and sequencing depths, the summary fastqc charts are shown from the paired end reads (R1 and R2) acquired from sample 92. Because of the decision not to deplete the host content, and in the context of patients with severe colitis providing some samples containing largely blood and little faecal material, reads from host material were over 50% in around 30 samples. A small number of these were noted to be almost entirely host content and very few or no microbial counts in the abundance tables. As a result of this, seven samples were excluded from abundance and functional pathway analyses.

R1



R2

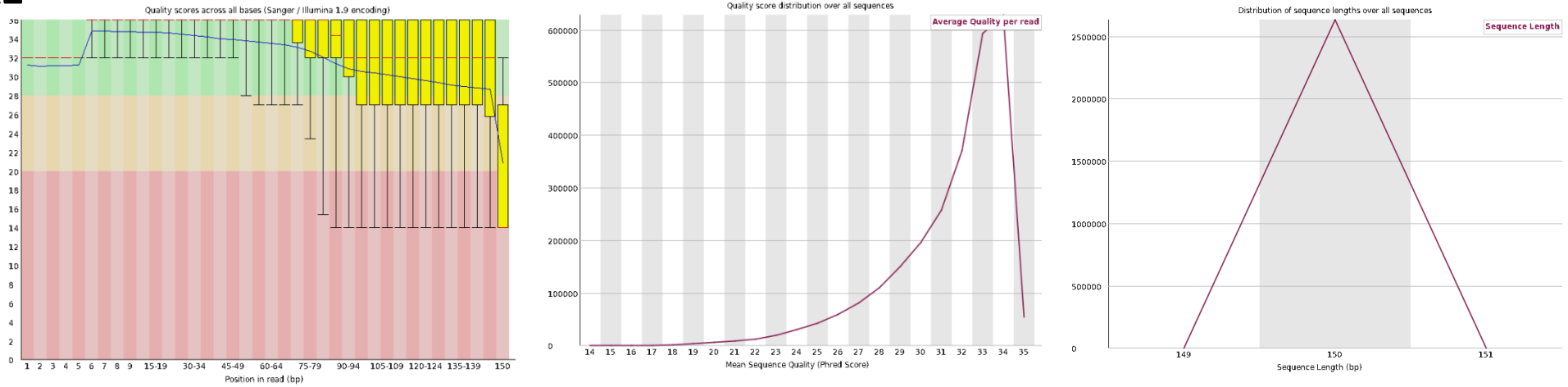


Figure 2 - 6: Exemplar quality scores and sequence length obtained from paired end shotgun metagenomic sequencing of faecal samples for IBD patients

The sequencing from this patient included 2.6 million sequences at 150bp per paired end read. This encompassed 394.7Mbp per reading.

Diversity analyses were undertaken using the 'Vegan' plug in for R (Oksanen et al., 2017). Differential abundance analyses were undertaken as per those described for the 16S dataset. Non-normalised data was taken from HUMAnN, with TSS normalisation utilised directly within MaAsLin2. The same rationale with regard to FCP utilisation for correction for baseline mucosal inflammation, with a composite measure again used unless otherwise stated, for example, if evaluating borderline signals in a smaller cohort with a FCP more closely associated with the time of sequencing. As before, an FDR of <0.2 was considered relevant, with scores of <0.05 highlighted by '***'. Minimum prevalence within the dataset for inclusion was set at 0.1 (i.e. 10%). For the shotgun analyses, this process was applied only to species and strain level data, to try to derive deeper understanding than achieved with the lower resolution 16S rRNA analysis.

2.5 Classifications, clinical indices and treatment response

Several different clinical indices are used through this thesis to classify disease subtypes and assess disease severity. For both UC and CD, the Montreal Classification is utilised (Silverberg et al., 2005). In Crohn's, the disease is differentiated by the locations where inflammation is present and the nature of that inflammation, whilst UC focusses on extent of inflammation and severity. In CD, clinical severity was determined using the Harvey Bradshaw Index (HBI), first developed in 1980 (Harvey and Bradshaw, 1980). Endoscopic severity was determined using the Simple Endoscopic Score for Crohn's Disease (SESCD) and is presented as the cumulative score from each segment (Daperno et al., 2004). Clinical and endoscopic severity in UC was determined using the Partial and

Endoscopic Mayo Scores (Schroeder, Tremaine and Ilstrup, 1987). The UC Endoscopic Index of Severity (UCEIS), the preferred method for scoring endoscopic severity, is not routinely used by non-IBD gastroenterologists and was not available for all patients. This was collected for all patients undergoing endoscopy with the inception team (Travis et al., 2013) or where image documentation was adequate for retrospective calculation. These scores are summarised in ***Table 2 – 3.***

Table 2 - 3: Clinical classifications and disease activity indices

Crohn's Disease Classifications and scores		
Montreal Classification		
Age A1: <16 A2: 17-40 A3: >40	**Location** L1: Terminal ileum L2: Colonic L3: Ileocolonic L4: Upper GI	**Behaviour** B1: Non-stricturing, non-penetrating B2: Stricturing B3: Penetrating p: Perianal
Harvey-Bradshaw Index - HBI (scored for the previous day). Score <5 remission, 5-7 mild, 8-16 moderate, >16 severe		
General wellbeing 0-4: range from 'very well' to 'terrible' **Abdominal Pain** 0-3: range from 'none' to 'severe'	**Number of Liquid stools** 1 point per bowel motion **Abdominal Mass** 0-3: range from 'none' to 'definite and tender'	**Complications** Score 1 point for any of: Arthralgia, uveitis, erythema nodosum, aphthous ulcers, pyoderma gangrenosum, anal fissures, new fistula, abscess
Simple Endoscopic Score for Crohn's Disease - SESCD (score per segment for ileum, right colon, transverse colon, left colon and rectum)		
Size of ulcers (diameter) 0 – 3: range from 'absent' to 'very large (>2cm)' **Ulcerated surface** 0 – 3: range from 'none' to '>30% of segment'	**Affected surface** 0 – 3: Range from 'none' to '>75% of the segment'	**Presence of narrowing** 0 – 3: Range from 'None' to 'Not passable, frank stenosis'
Ulcerative Colitis Classifications and Scores		
Montreal Classification		
Extent E1: Proctitis E2: Left sided (not beyond splenic flexure) E3: Extensive (beyond splenic flexure)	**Severity** S0: Remission, no active inflammation S1: Mild S2: Moderate S3: Severe	
Partial Mayo; Score 0 – 1 remission, 2 – 4 mild, 5 – 6 moderate, 7 – 9 severe		
Stool frequency 0 – 3: range from 'normal' to '5 or more than normal'	**Rectal bleeding** 0 – 3: range from 'no blood' to 'blood alone passed'	**Physician's global assessment** 0 – 3: range from 'normal - subscores mostly 0' to 'Severe – subscores are mostly 2 – 3'.
Endoscopic Severity; Endoscopic Mayo Score and The Ulcerative Colitis Endoscopic Index of Severity (UCEIS)		
Endoscopic Mayo 0: Inactive; no endoscopic change 1: Mild; erythema, decreased vascular pattern 2: Moderate; marked erythema, absent vascular pattern, friability, erosions 3: Severe; spontaneous bleeding, ulceration	**UCEIS** **Vascular pattern:** 0 – 2 from 'normal' to 'obliteration' **Bleeding:** 0 – 3 from 'none' to 'luminal moderate or severe' **Erosions and ulcers:** 0 – 3 from 'none' to 'deep ulcer' **Remission** 0 – 1 **Mild** 2 – 4 **Moderate** 5 – 6 **Severe** 7 - 8	

When analysing data, both in terms of galectins and microbiome datasets, patients have been stratified in several ways. The first approach was to stratify baseline patient data into groups according to subsequent treatment responses at 6- and 12-month timepoints. Patients were separated patients into those obtaining ‘remission’, those having a ‘partial response’ and those in whom ‘no response’ was observed. Due to the ‘real-world’ delivery of this work in an NHS environment under significant strain, it was necessary to apply a hierarchy to these assessments to prevent the exclusion of excessive patient numbers due to missing data. Where available, endoscopic or radiological (MR / CT enterography in those with isolated small bowel disease) assessments were utilised. Where this had not been undertaken, biochemical surrogates, faecal calprotectin and CRP, were employed. If these too had not been obtained, but clinical assessment had been documented, the outcomes from these assessments were used. Similarly, if a UC patient had a Partial Mayo score of 6 but colonoscopy and biopsies at the same time had shown no disease activity, the colonoscopy would be used to decide the activity assessment and the Partial Mayo disregarded.

It must be acknowledged that the use of clinical or biochemical assessments in isolation leaves room for error, particularly in the case of isolated clinical assessments in Crohn’s disease (Falvey et al., 2015). As reflective of our local clinical practice, driven by an backlog in endoscopy, faecal calprotectin samples were taken wherever possible. To try to minimise any inaccuracy from the use of surrogate markers, a more stringent calprotectin cut-off of 150ug/g was utilised rather than 200ug/g as seen in recent benchmark studies in Crohn’s disease (Noor et al., 2024). This same threshold was used in UC patients given that it aligned with

historic thresholds (Gisbert et al., 2009) and more recent validation studies (Hart et al., 2019). Chosen clinical and endoscopic thresholds align with the initial derivation studies for the given scores (as shown in ***Table 2 – 3***) and whilst less stringent from the criteria typically seen in phase 3 clinical trials, were feasible to collect during standard clinical follow up. The criteria utilised can be found in ***Table 2 – 4.***

Table 2 - 4: Criteria utilised to determine treatment response

Response Grading	UC	Crohn's
'Partial response' criteria		
Partial clinical response	≥30% in Partial Mayo **OR** Fall by ≥3 points	Fall in HBI of ≥3
Partial biochemical response	Fall in FCP >50% **OR** Fall in CRP >50%	Fall in FCP >50% **OR** Fall in CRP >50%
Partial mucosal response	Fall in UCEIS >50% from baseline * If only endoscopic Mayo given, fall ≥1 from baseline	Fall in SESCD >50% from Baseline **OR** Improvement on imaging from baseline
'Remission' criteria		
Clinical remission	Partial Mayo <3 No individual score >1 No steroids or treatment escalation planned or undertaken within 3 months	HBI <5 No steroids or treatment escalation planned or undertaken within 3 months
Biochemical remission	CRP <5 AND FCP <150	CRP <5 AND FCP <150
Mucosal remission	Mayo 0 or UCEIS ≤1	SESCD <3 **OR** No active disease on imaging

HBI = Harvey Bradshaw Index, FCP= Faecal Calprotectin, CRP= C-Reactive Protein, SESCD = Simple Endoscopic Score for Crohn's Disease

Where patient numbers were under powered to demonstrate significant difference across three groups, patient assessments at the 6- and 12-month timepoints were re-classified into ‘responders’ and ‘non-responders’. ‘Responders’ were those who met any of the response or remission criteria in ***Table 2 – 3***, whilst those who did not were ‘non-responders’. This approach allowed analyses to focus in on those with the most treatment resistant disease, in whom earlier treatment escalation would be beneficial. It also aligned with benchmark studies in the microbiome field where patients were stratified, for example, as ‘remission vs no remission’ and ‘colectomy vs no colectomy’ (Schirmer et al., 2018).

Finally, when analysing the galectin data, multiple samples were obtained often outside of the 6- and 12-month time points. In these analyses, it was important to know how the disease status at the time of sampling influenced serum galectin level and whether the results could separate out these patients. For these analyses, the disease is stratified as ‘Active’ or ‘Inactive’. Any patient not meeting the remission criteria above (HBI ≥5 or Partial Mayo ≥3, SESCD ≥3 or UCEIS >1, CRP ≥5, FCP ≥150) was determined to still be in an active disease state. The previously described hierarchy was again employed for this. This approach was also replicated when analysing the IBD Disk data.

CHAPTER 3: Delivering ‘Rapid Access’ to IBD Diagnosis and Characterising an Inception Cohort

3.1 Abstract

Background:

The prevalence of IBD is increasing. Delays to diagnosis result in adverse outcomes. Late presentation, primary care delays and, amidst increasing health service pressure, secondary care delays are all contributing to this, particularly in our trust. We have established a bespoke rapid access diagnostic pathway and present data on how best to combine available clinical tools for early diagnosis and efficient allocation of clinical resources.

Methods:

A rapid-access pathway with dedicated clinic and endoscopy resources was implemented for suspected IBD, with patients triaged according to symptoms and FCP. Detailed clinical indices, repeat FCP results, a standardised 13-point clinical history and PROMs were collected prospectively as patients progressed through the diagnostic pathway.

Results:

From Jan 2021 to September 2023 the pathway saw 767 patients, with 423 diagnosed with IBD. Time from urgent GP referral to review fell from 84 to 32 days and fell to 14 days once the referral reached the inception team. Median time to diagnosis and treatment initiation was 48 days in IBD. Despite this, patients with CD reported a median 10 months of symptoms by first clinic appointment.

The most frequent symptoms at presentation in CD were abdominal pain (94%), looser stools (84%) and fatigue (79%), whilst in UC it was per-rectal bleeding (94%), urgency (82%) and looser stools (81%). Strongest IBD predictors were blood mixed with stools (CD odds ratio [OR] 4.38; 95% CI 2.40-7.98, UC OR 33.68; 15.47-73.33)

and weight loss (CD OR 3.39; 2.14-5.38, UC OR 2.33; 1.37-4.00). Repeat FCP testing showed reduction from baseline in non-IBD. Both measurements >100ug/g (AUC 0.800) and >200ug/g (AUC 0.834) collectively predicted IBD. However, a second value ≥220ug/g considered alone, regardless of the first result, was more accurate (Youden's Index 0.74, AUC 0.923). Modelling symptoms with FCP increased AUC to 0.947.

Elevated baseline IBD disk in UC predicted the subsequent need for advanced therapies (AT) (p=0.014) and inpatient treatment (p<.001). After diagnosis, ongoing disability was driven by persistent active disease. A Disk '*Emotions'* domain scores ≥7 had a 100% sensitivity for moderate-severe depressive symptoms on HADS.

Conclusion:

Dedicated clinical resource and focussed triage and prioritisation can transform the path to IBD diagnosis, even in a healthcare system under huge strain. Serial FCP measurement prevents unnecessary colonoscopy but modelling this with clinical symptoms increases the predictive ability further. In the era of home FCP testing, and with further validation, this combination could form the basis of effective self - referral pathways. The IBD disk can accurately identify clinically relevant depressive symptoms and help predict treatment outcomes in the first-year post IBD diagnosis.

3.2 Introduction

3.2.1 The impact of delayed diagnosis in IBD

The importance of early diagnosis in IBD is well established. A recent systematic review and meta-analysis by the POP-IBD group (Jayasooriya et al., 2023) demonstrated that delayed diagnosis is associated with adverse outcomes in both UC and CD. In CD, delayed diagnosis was associated with higher likelihood of both stricturing (OR 1.88; CI 1.35-2.62) and penetrating (OR 1.64; CI: 1.21-2.20) disease, translating to an increased risk of surgery (OR 2.24; CI 1.57-3.19). In UC, though more overt symptoms guarding against delayed diagnosis, where present it is associated with a significantly increased risk of colectomy (OR 4.13; CI: 1.04-16.40). A collated median of all articles included in this review found the median time to diagnosis to be 8 months in CD (IQR 5.0-15.2) and 3.7 months in UC (IQR 2 – 6.7). However, all datasets included in this review were generated from clinical outcomes prior to the advent of COVID-19. There are few large IBD cohorts recruited during this time where impact of COVID upon time to diagnosis has been explored. In more general terms, studies from both Europe (Splinter et al., 2021) and North America (Czeisler et al., 2020) demonstrate increase in healthcare avoidance even in those with alarm symptoms during COVID19. Datasets demonstrating the longevity of this impact are less forthcoming. Multiple studies in oncology have addressed the issues of diagnostic delays in the UK and the associated negative impact on healthcare outcomes, including modelling work from close to 25 000 patients diagnosed with colorectal cancer who may have shared many presenting symptoms with IBD patients (Maringe et al., 2020).

3.2.2 Points of delay within IBD care pathways

Though the lack of IBD specific data is noted, it is clearly demonstrable that both during and following on from COVID-19, NHS waiting times have spiralled. A 2023 report (Pope, 2023) highlighted the increase from 4.4 to 7.1 million patients waiting for NHS diagnostics, treatment decisions and procedures between February 2020 and August 2022. National data regarding referral to treatment waiting times in 2023 (NHS England, 2023) showed that only 58% of patients waiting for treatment are within 18 weeks, against a national target of 92%. This, falling against the backdrop of the steadily increasing incidence and prevalence of IBD, (King et al., 2020) raises the possibility of significant secondary care delays, both in receiving an initial outpatient appointment and thereafter undergoing the necessary diagnostic tests.

Before the patient reaches secondary care services, there are two other points of significant delay. Firstly, the POP-IBD group (Blackwell et al., 2021) went on to demonstrate, via a case-control study, that patients subsequently diagnosed with IBD have a significant excess of gastrointestinal symptoms as far back as five years prior to diagnosis (9.6% in UC, 10.4% in CD, 5.8% in controls). Qualitative work by another group explored reasons why such a long lead in to seeking medical attention may exist (AWARE-IBD Diagnostic Delay Working Group, 2024). Major contributions were ascribed to the misattribution and normalisation of significant symptoms, family and work pressures, fear and embarrassment and poor availability of face-to-face primary care review. Secondly, the POP-IBD group identified further delays that occur once patients have sought primary care review, with up to 25% of patients reporting symptoms to a primary care physician more than 6 months prior to diagnosis in this study. Pre-existing diagnoses of IBS (HR 0.77; CI: 0.60 – 0.99) and

depression (HR 0.77; CI 0.60-0.98) were found to have significantly increased the risk of delay to specialist review. Vavricka (2012) were also able to demonstrate disease specific predictors or delay, with ileal disease distribution being independently associated with increased risk (OR 1.69, p 0.025). These points of delay are summarised in ***Figure 3-1.***

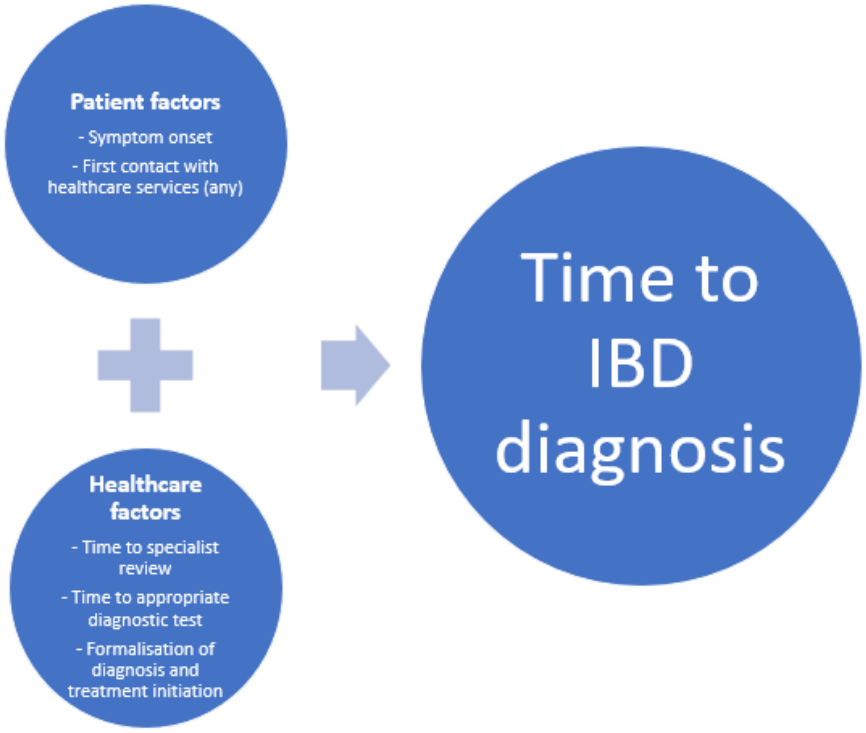


Figure 3 - 1: Points of delay to IBD diagnosis

It is recognised that multiple points of delay may lead to late diagnosis in IBD. This process starts when there is a delay to seeking medical opinion after developing symptoms of IBD. Healthcare factors start after an individual's first presentation to a healthcare provider, whereby failure to interpret symptoms as compatible with IBD may lead to no investigation being undertaken. This risk remains even after referral to secondar care. Once a decision is taken to investigate, growing waiting list or procedural issues such as inadequate bowel preparation may further exacerbate the issue. *Figure adapted from Jayasooriya et al., 2023.*

3.2.3 What should we aim for?

The 2019 IBD UK National report identified delay to diagnosis as a major challenge facing IBD patients (IBD UK, 2019). To combat secondary care delays, UK IBD consensus guidelines would suggest that any patient referred with suspected IBD should be seen within four weeks of that referral being made (Lamb et al., 2021). The IBD Standards guidance (IBD UK, 2020) agree with this timeframe, whilst both emphasise that in some patients four weeks may be too long to prevent hospital admission. Both references highlight the need to agree pathways between primary and secondary care for referral which should utilise adjuncts such as FCP to guide primary care physicians as to when a referral should be made. Finally, the IBD standards also elucidate the need for a holistic and detailed assessment at first review, inclusive of nutrition, bone health and mental health. Integration with the wider IBD multidisciplinary team should be present from the outset.

These reports and guidelines have been followed by national campaigns including ‘Getting it Right the First Time’ (NHS England, 2022) and ‘Cut the Crap’ (Crohn’s and Colitis UK, 2023). Indeed, time to diagnosis has been established as a key performance indicator for IBD care (Quraishi et al., 2023). The latest IBD UK report (IBD UK, 2024) builds on this further, highlighting rapid diagnosis as a crucial step in preventing long-term complications and bringing in desired limits to waiting times across all stages of the diagnostic pathway for those with suspected IBD. Despite all this effort, additional funding has not been made available and expanding services in an adverse healthcare environment is fraught with difficult. As such, there must also be better utilisation of existing resources.

3.2.4 Existing tools clinical tools to aid in the diagnosis of IBD.

FCP is an established tool in the diagnosis of IBD. First evaluated over 30 years ago (Roseth et al., 1992), calprotectin is a cytoplasmic protein found within neutrophils. Colonic mucosal inflammation results in neutrophil activation and consequent calprotectin release, with reported stability in the faeces for up to 5 days (Dhaliwal et al., 2015). Its use has previously been shown to reduce time to diagnosis and it forms a central cog in most referral pathways (Hicks et al., 2020 and Lamb et al., 2019). Despite this, and its use in clinical practice for over a decade, there is no consensus agreement about all aspects of its implementation. Owing to variations in commercially available assays, current consensus guidelines (Lamb et al., 2021) do not specify a single cut-off, instead suggesting a range between 100-250ug/g. It is also known that FCP can be transiently elevated due to medications or several non-IBD conditions. Common causes of ‘false’ positives include gastrointestinal infections, diverticular disease, colonic polyps, colorectal cancer, GI bleeding, non-steroidal anti-inflammatory drugs, proton pump inhibitors and obesity (D’Amico et al., 2020). Implementation studies (Turvill et al., 2018) and previous meta-analyses (Waugh et al., 2013) in IBD hint at the value of repeat testing (typically after an interval of 4 weeks) to increase specificity. This is yet to be mandated in national guidance. This in part owes to the need to offer primary care physicians a route for early referral in those with significant symptomatology and FCP elevation (even if infection has not been excluded and the history is short). The extreme pressure on primary care at present means that practice is highly variable, and capacity is seldom available for further GP led review in the community and repeat FCP testing pre-referral. We are left with an environment where FCP (and perhaps moving

forwards, Faecal Immunochemical Testing [FIT]) is a primary tool driving referral to secondary care (Macpherson et al., 2021, White and Makin, 2022, Lee et al., 2023) . Given these are the patients attending our new patient clinics, are there other tools that can be coupled with FCP to help further predict a subsequent IBD diagnosis?

The value of the clinical history is often overlooked. Symptom prevalence and predictive ability (for an IBD diagnosis vs non-IBD) never been formally evaluated in a prospective study. Within the UK, the last study (Sawczenko et al., 2003) to prospectively collate the presenting features of IBD is 20 years old and dealt only with paediatric patients. Whilst symptom prevalence was addressed in this cohort, it did not focus on the ability for individual symptoms to discriminate IBD. A large retrospective study utilising primary care databases did attempt to do this for both IBD and colorectal cancer (CRC) (Stapley et al., 2017). This work included 1661 new diagnoses of CRC and 9578 new diagnoses of IBD, each matched with three controls. Taking individual symptoms, the strongest predictors of CRC/IBD were ‘Change in Bowel Habit’ and ‘Rectal bleeding’. In a multivariable analysis, the same symptoms were most strongly associated with CRC/IBD. By combining two symptoms as a predictor, diarrhoea in the presence of rectal bleeding enhanced the predictive capacity seen. This paper did not split UC and CD and in doing so emphasises symptoms typical of UC but misses the opportunity to aid CD diagnosis. In North America, the OSCCAR cohort published a descriptive analysis of the symptoms of 233 CD and 150 UC patients at presentation between 2008 – 2013 (Perler et al., 2019). In CD, the commonest symptoms were found to be fatigue (80.6%) and abdominal pain (80.4%). In UC, passing blood with bowel motions

(86.6%) and loose stools (86.5%) were the most common. Principal Component Analysis (PCA) was utilised to cluster symptom components into distinct phenotypes at presentation (using a clinically unrealistic 41-question symptom inventory). The authors made no attempt to contrast presenting symptoms with those in whom IBD was subsequently excluded. Though other studies characterise symptom burden against disease course (Singh et al., 2011) there are few others that assess presenting phenotype in this way. No studies present the predictive ability of FCP alongside clinical symptoms.

3.2.5 Patient Reported Outcome Measures (PROMs) in IBD

PROMs are tools that have been developed across multiple settings, typically as questionnaires, that allow patients to directly report the severity and impact of their disease. ‘Quality of life’ is a key construct within overall health and its improvement a key target of treatment. However, it is a construct seldom adequately observed using standard clinical indices. The correct use of PROMs, as defined by the United States Food and Drug Administration (U.S Department of Health and Human Services Food and Drug Administration, 2009) should allow patients to report symptoms or responses to set questions without any input from the medical professional with whom they are interacting or contribution from other disease activity indices. In recent years the development of new tools has been a major focus to improve our understanding of the holistic impact a disease process has on quality of life (Fletcher et al., 2021). To ensure the tools utilised are robustly assessed and of sufficient quality to achieve there aims, a few initiatives have been developed including The Consensus Based Standards for the Selection of Health Measurement Instruments

or COSMIN (Mokkink et al, 2014). A recent systematic review of the COSMIN database identified 21 different IBD specific PROMs that have been described in published systematic reviews (Fletcher et al., 2021). Another review, undertaken with less robust inclusion criteria and outside of the COSMIN database identified 44 IBD related PROMs (Van Andel et al., 2020). Whilst many PROMs have been described, not all have undergone robust validation in a clinical setting.

3.3 The local climate

Multiple performance criteria deteriorated significantly at University Hospitals Birmingham NHS Foundation Trust (UHB), throughout COVID and beyond. General outpatient delays within Gastroenterology ran at around 12 weeks for urgent referrals and up to 52 weeks for those classed as routine during the majority of this study period. That is just the delay for first outpatient review. Within endoscopy, GP referrals for straight to test colonoscopy typically took 8-10 weeks, though that has been a key target for improvement latterly. During the study period, GP straight to test referrals were often prioritised at the expense of internally requested procedures, meaning patients seen in clinics were delayed further.

This data is to an extent anecdotal and experiential. It is however backed up by national figures from 2023 (NHS England, 2023). Within Gastroenterology, nearly two thirds of patients (61.1%) waiting for treatment had done so more than 18 weeks. Of those who have received treatment, only half received this in less than 24 weeks. Prior to the implementation of the ‘IBD Inception pathway’ all patients

referred with suspected IBD would have been seen within this outpatient pool, some that would have been associated with appreciable harm for some.

3.3.1 The desired model of care

Any pathway operating in the current environment relies upon the early identification of those requiring rapid review, ring fenced resource to deliver both initial assessment and early endoscopy, adequate time to provide holistic assessment and pathways for early escalation of care and integration within the multidisciplinary team. With those as central tenets, I have supported the development of the following model of care. I have personally implemented and delivered this from the outset, delivering initial triage, clinical review, diagnostic colonoscopy, and treatment for patients seen at QEHB since January 2021. The final model presented below has evolved according to the need for increased capacity and from learning in its early delivery.

FCP is mandated as part of the referral process to support primary care colleagues with whom to refer and allow for identification of suspected IBD early in the disease course. The senior consultant body is responsible for referral triage, with cases passed directly to the IBD fellow at that point to allow telephone triage, prioritisation and appropriate booking. The same process applies to the utilisation of ring-fenced endoscopy slots, also delivered by the IBD fellow to allow for continuity of care through the diagnostic process. Patients seen in this clinic were primarily those in whom IBD was suspected. However, patients who underwent two-week wait colonoscopy for suspected colorectal cancer and were subsequently found to have

IBD were passed to Inception team for initial review and treatment. Once diagnosis is established, treatment should be commenced on a same day basis. Whilst this was standard for patients undergoing colonoscopy in our ring-fenced lists, it was not always possible for those having two-week wait colonoscopy with external providers (insourcing companies paid by the trust to provide diagnostic services). Early access to this ensured that treatment delays did not occur.

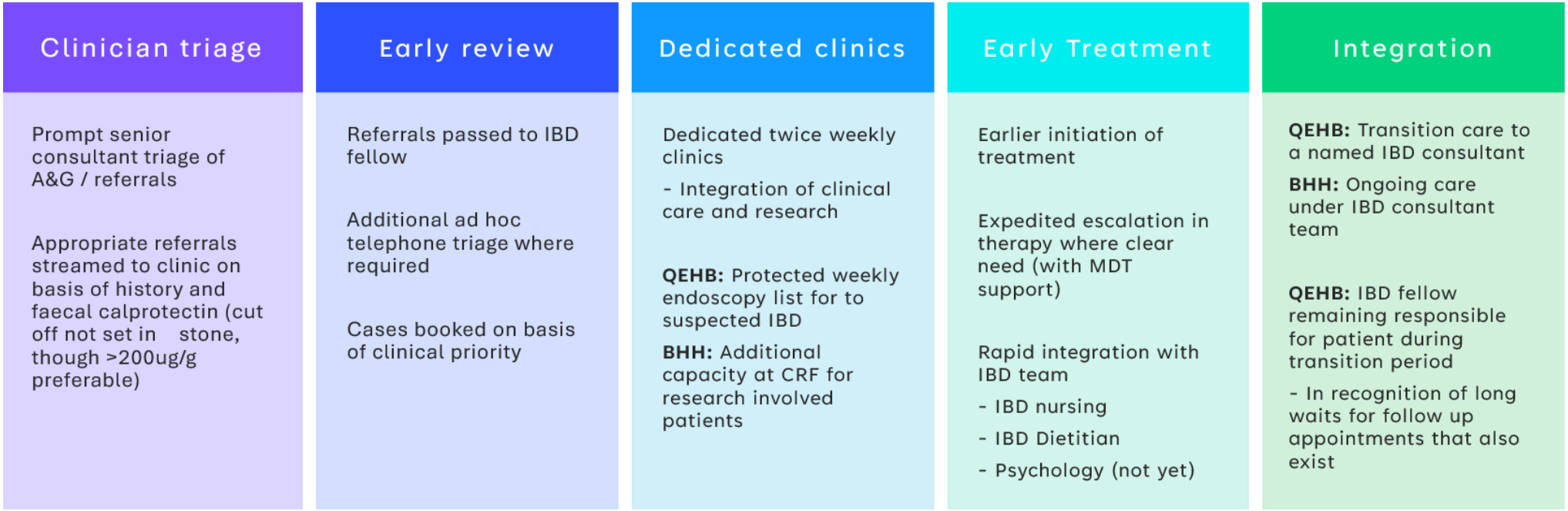


Figure 3 - 2: The IBD Inception Model

The desired model of care at UHB. The process should start with early identification of appropriate referrals, rapid transfer to the IBD team who then undertake further triage, including ad hoc telephone assessment, to prioritise clinic appoints and subsequent endoscopy according to clinical need at the earliest stage possible. Once diagnosis is obtained treatment is initiated same-day and rapid escalation in therapy is sought where required. Once stabilised, patient care transitions to an individual named consultant. QEHB = Queen Elizabeth Hospital Birmingham. BHH = Birmingham Heartlands Hospital.

With careful integration with nursing colleagues, high risk patients are signposted for early review and supported education regarding the diagnosis. In turn, nurses have a single point-of-access for queries regarding newly diagnosed patients in the form of the IBD fellow. Where necessary, care can be progressed rapidly with prioritisation of ‘biologics’ clinic and infusion unit slots for those most in need of AT. Though difficult to quantify, the ability to progress care at this rate could prevent hospital admissions.

3.3.2 Pathway Implementation

The clinical pathway was initiated in January 2021. Through early successes in its implementation and growing demand, it has been possible to double the clinical capacity at Queen Elizabeth Hospital (QEHB) and roll out a duplicate model of the clinic at Birmingham Heartlands Hospital (BHH), a process I have heavily supported. The trust has made long term adaptions to care deliver that place the inception pathway at the centre of its model for IBD care.

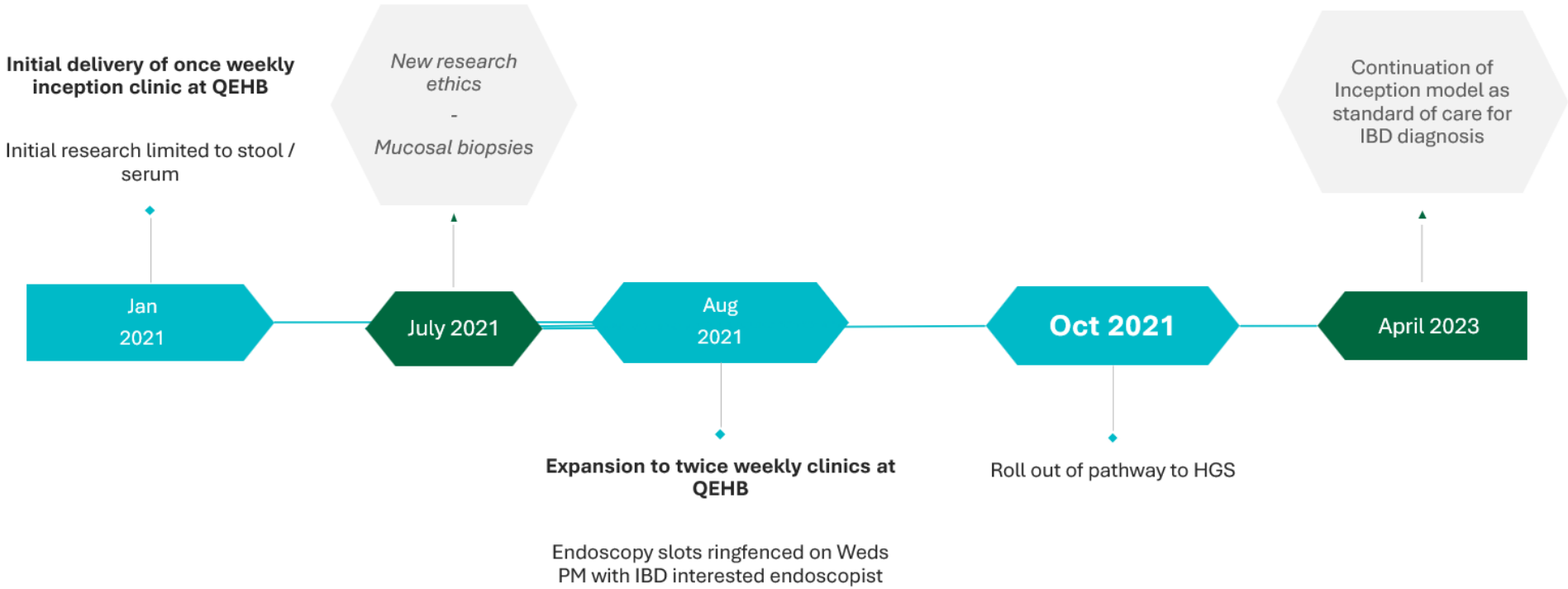


Figure 3 - 3: Timeline for Implementation and evolution of IBD Inception pathway at UHB

Initial anticipated throughput allowed me to deliver clinic slots for 3 patients and 3 colonoscopy slots per week. Given existing backlogs and high clinical throughput, this was rapidly expanded. Research has been integrated with the pathway from the outset. This process is now standard care across all trust sites after its implementation across Birmingham Heartlands Hospital, Good Hope Hospital and Solihull Hospital.

3.4 Clinical throughput and pathway performance

As of September 2023, a total of 767 patients had been seen across the two IBD inception clinics since the initial implementation in January 2021. I personally had seen 520 of these. At the time of writing, a final diagnosis was available for 762 of these patients. 215 were diagnosed with UC, 208 CD and 339 with assorted non IBD-diagnoses.

Considering the overall trust performance of 12 weeks for an urgent new patient clinical appointment and potentially months subsequently waiting a colonoscopy requested from an internal source, the key performance indicators for the clinic in terms of time to review are displayed in ***Table 3-1***. Outside of symptom duration which has been reliably collated across sites, data collection at BHH has been sporadic and outside of my control.

Table 3 - 1: Inception Clinic Key Performance Indicators

	Crohn's Disease	**UC**	**Non IBD**
	All values median (IQR)		
Duration of symptoms, ***months*** (n= 713)	10 (19)	4 (8.5)	6 (9)
GP ref to review, **days** (n=427)	32.5 (36.25	29 (35.5)	33 (27.25)
Passed to Inception to review, **days** (n=603)	14 (11)	13 (15)	18 (12)
Review to endoscopy, **days** (n=301)	23 (24.5)	16 (25)	32 (37)
GP ref to treatment, **days** (n=186)	56.5 (85.5)	46 (52.5)	
First outpatient review to biologic start, **days** (n=104)	128 (162.25)	140 (177)	

As shown, there remain significant patient and primary care related barriers to timely diagnosis, particularly in CD. In this group, duration of symptoms is significantly longer and on average runs around 10 months ***(Figure 3-4)***.

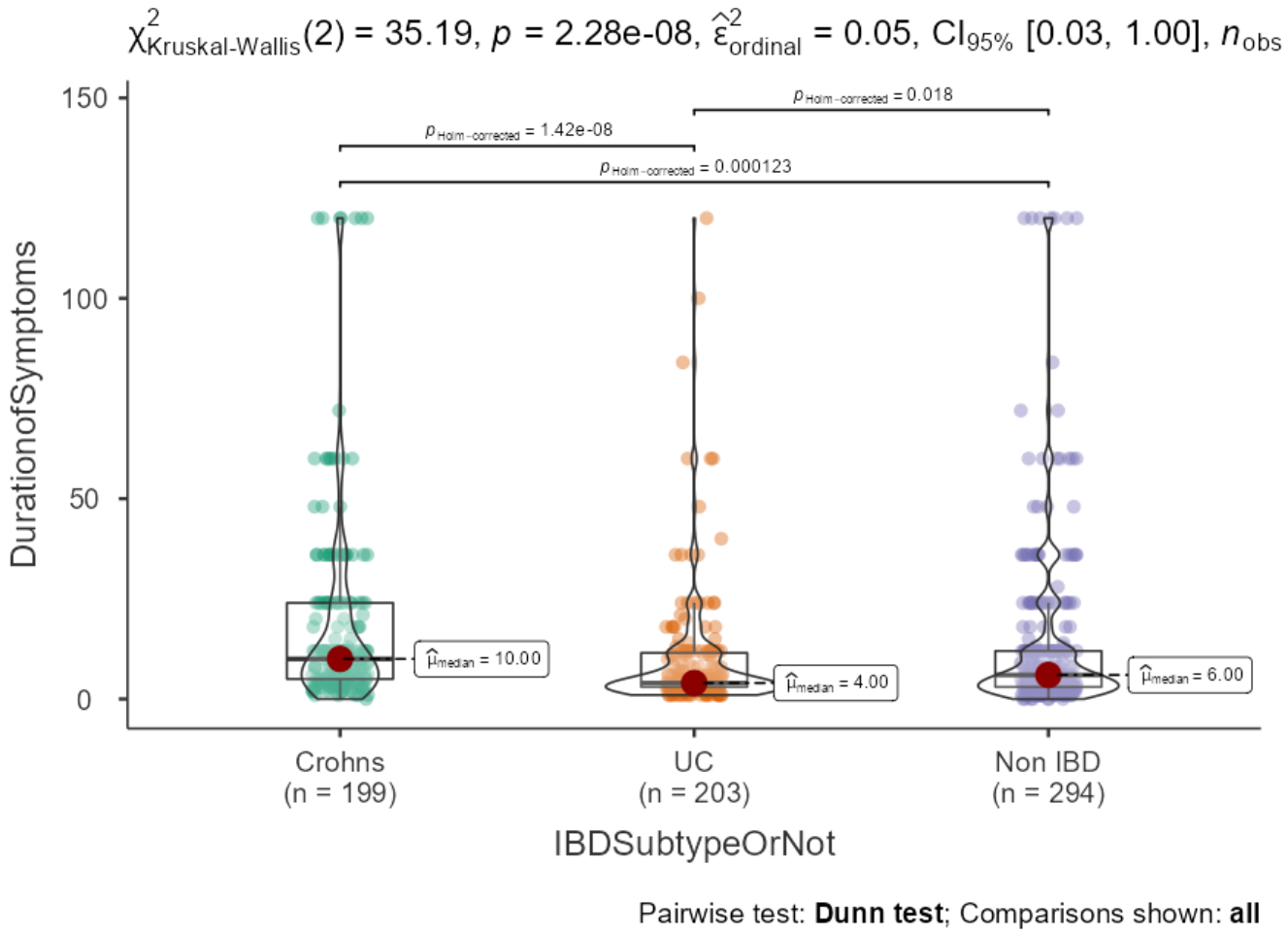


Figure 3 - 4: Duration of Symptoms (m) at the time of first outpatient review, split by diagnosis.

From our cohort, 696 patients had a documented patient reported symptom duration at first review. Patients who go on to review a diagnosis of Crohn's disease (n=199) have a significantly longer duration of symptoms than those with either Ulcerative Colitis (n=203) or a subsequent Non IBD (n=294) diagnosis. This remains highly significant despite correction for multiple comparisons (Kruskal-Wallis. [2], Dunn test p_{holm} 0.02 for non IBD, p_{holm} 0.0001 for UC).

When compared to overall waiting times, the implementation of our care pathway has been able to markedly reduce overall delays, both in terms of outpatient review (up to 84d for urgent referrals vs median 32d via the inception pathway for all diagnoses). The benefit is also borne out across waiting times for endoscopy, which are reduced in those subsequently found to have IBD vs those that are not (Mann-Witney U 7934 $p<0.001$). In fact, patients diagnosed with IBD are seen, diagnosed and treated in close to half the time urgent gastroenterology referrals wait for their first appointment (48 vs 84 days).

Once received by the Inception team, those subsequently diagnosed with IBD are seen within two weeks. The value of early clinical triage is seen at this stage, with those going on to be diagnosed with IBD given clinical priority and seen sooner than those in whom IBD was later excluded ***(Figure 3-5)*****.**

Furthermore, patients with CD subsequently found to have more severe disease had been seen more quickly prior to their procedure (based on SESCD and correlation with time to first outpatient review, n=123, $r_s = -0.302$, $p<0.001$). The same applied to those with more severe disease in UC, this time determined by UCEIS score (n=81, $r_s = -0.281$ $p=0.011$).

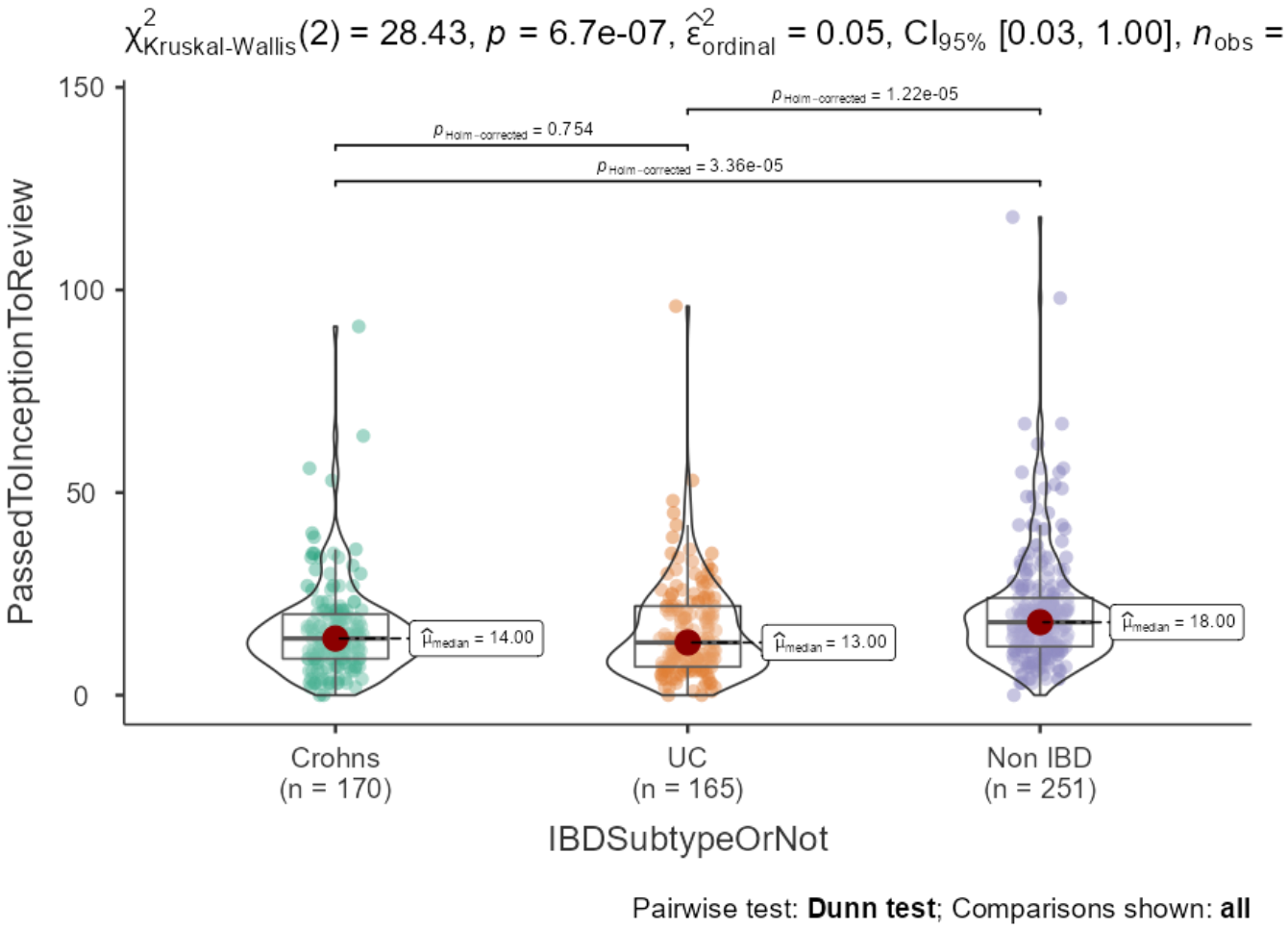


Figure 3 - 5: Time to review after referral triaged by Inception team.

This analysis was undertaken in the cohort seen in QEHB where data was prospectively collected. Whilst delays to initial consultant A&G response even out the differences, once the referral has been passed on to the IBD inception team, detailed clinical triage is able to pull out those who go on to be diagnosed with IBD and bring them to clinic earlier. There is a significantly shorter wait for those subsequently diagnosed with UC (n=165) or Crohn's (n=170) than seen in those with Non IBD diagnoses (n=251). Kruskal-Wallis[2], Dunn test p_{holm} 0<.001 for UC, p_{holm} <.001 for CD.

3.4.1 The Clinical Cohort

The overall demographics are presented in ***Table 3-2***. For IBD patients, Montreal classifications are presented. For the grouped 'Non-IBD' cohort, a broad breakdown of diagnoses is given. Whilst some in this cohort had significant organic pathology I have kept them grouped in this way as it reflects the case mix and diagnostic challenges seen in day-to-day practice.

Table 3 - 2: Clinical Cohort Demographic Overview

	Crohn's (n = 208)	**UC (n = 215)**	**NonIBD (n = 339)**	**Global test** Chi-sq[1] Kruskal-Wallis[2]
Age, median (IQR), y	34 (22)	37 (22.5)	35 (20)	p=0.08[2]
BMI, median (IQR), y	25.3 (9.53)	25.5 (6.18)	27.9 (10.4)	p=<.001[2]
Sex, % male	43	60	40	p=<.001[1]
Ethnicity, %				
White	67	66	74	p=0.004[1]
Asian	25	30	17	
Black	8	4	9	
Location (or grouping), n (%)	Ileal (L1) 91 (44) Colonic (L2) 59 (28) Ileocolonic (L3) 58 (28) Perianal 22 (11)	Proctitis (E1) 69 (32) Left sided (E2) 83 (39) Extensive (E3) 63 (29)	Transient infection 54 (16) Functional 171 (50) Other GI 114 (34)	
Behaviour / severity, n (%)	Non-stricturing, non-penetrating 172 (83) Stricturing 31 (15) Penetrating 5 (2)	Mild 87 (41) Moderate 99 (46) Severe 29 (13)		

3.5 What drives delayed presentation in IBD?

3.5.1 Duration of symptoms at index review

All patients were asked when they first started to experience gastrointestinal symptoms and the time interval to the date of outpatient clinic review was calculated. We have already demonstrated the significant increase in symptom duration in those going on to be diagnosed with CD ***(Figure 3-4)***. Looking at demographic factors across the cohort, trends relating to ethnicity did not reach statistical significance, though median symptom duration was longer in patients of black ethnic backgrounds (n=706 [White=486 median=6m, Asian=167 median=6m, Black=53 median=12m. Kruskal-Wallis (2) 4.22 p 0.121, no significant difference on pairwise testing). There was also no correlation between age and duration of symptoms (n=645, Spearman's Rho r_s -0.040 p 0.307). Outside of secondary care time frames, exploration of the drivers of delayed presentation or referral are beyond the scope of this chapter.

3.5.2 What are the consequences of delayed diagnosis in our IBD cohort?

In previous studies (Vavricka, 2012), ileal disease has been identified as a risk factor for delayed diagnosis in IBD. However, within this cohort, the location of CD activity did not impact upon symptom duration at diagnosis (n=199, L1 median 8.5m, L2 11.5m, L3 11m, Kruskal-Wallis (2) 0.07 p 0.96). However, within this same cohort, patients presenting with an increased duration of symptoms were significantly more likely to present with stricturing or penetrating complications than those who had uncomplicated disease ***(Figure 3-6)***. An early but currently not significant increase in symptom duration is seen in the 7 patients within the CD cohort who have progressed to surgery. (n=141, Median 12m vs 9m, Mann-Whitney U 654, p 0.078).

In those with UC, duration of symptoms did not associate with adverse outcomes. Those with more severe disease typically had a more fulminant presentation. Generally, those with more extensive disease (Montreal classification), trended towards earlier presentation (n=203, E1 median 5m, E2 4.5m, E3 4m, Kruskal-Wallis (2) 4.66 p 0.097, no significant pairwise tests). Similar patterns were seen when dividing the cohort by disease severity (S1 median 6m, S2 4m, S3 3m, Kruskal-Wallis (2) 4.95 p 0.084, no significant pairwise differences).

Across IBD subtypes, there was no significant difference in the duration of symptoms at presentation between those who did and did not require AT (All IBD, n=323, AT yes median 8m, AT no 5m, Mann-Whitney U 1300 p 0.117).

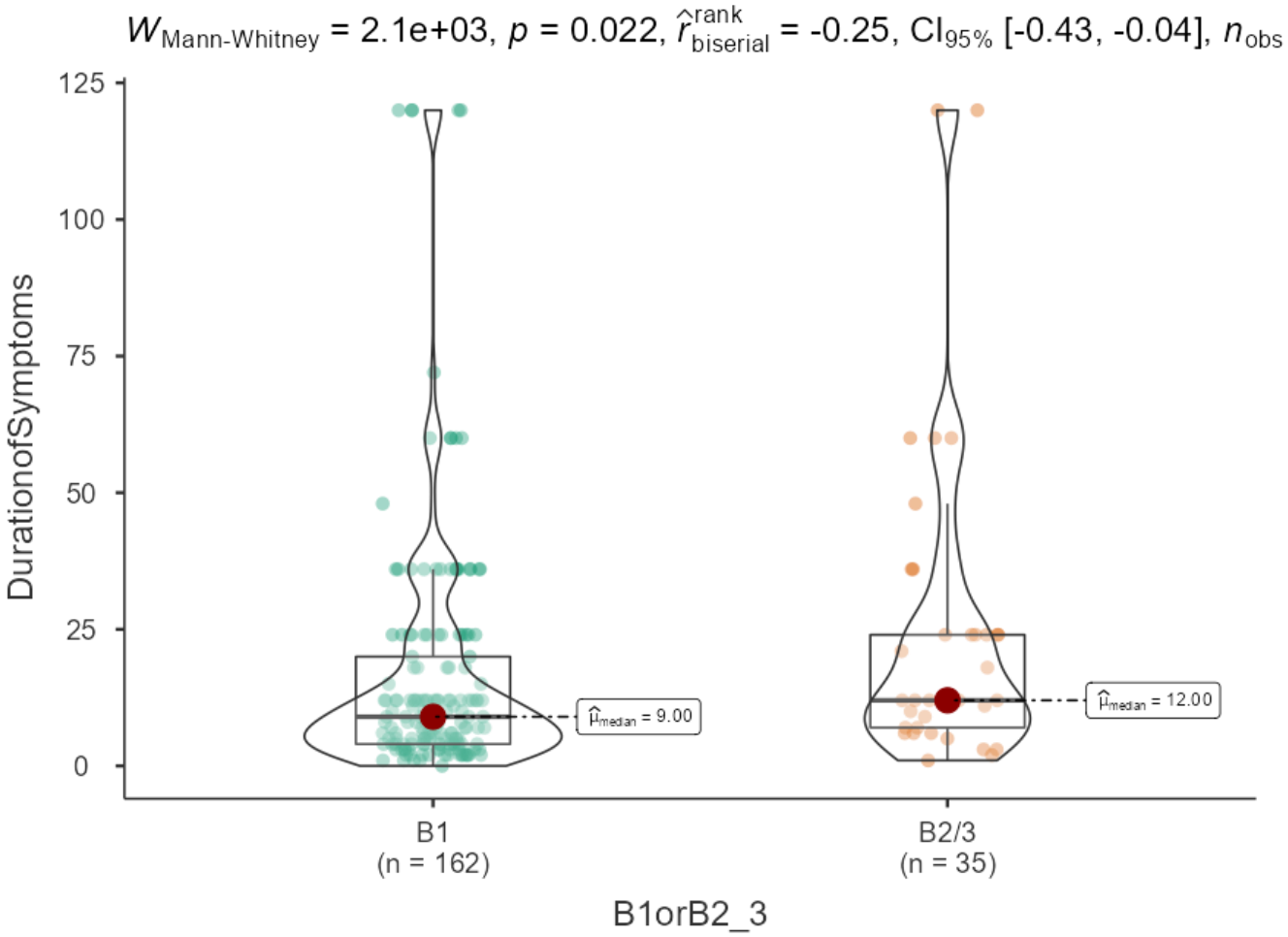


Figure 3 - 6: The Difference between duration of symptoms in those presenting with uncomplicated and complicated Crohn's disease.

Patients who have stricturing or fistulation complications at presentation have a longer duration of excess gastrointestinal symptoms prior to diagnosis (Montreal B1 n=162 vs B2/3 n=35, Mann-Whitney 2100, p 0.022). Despite grouping patients with Montreal B2 and B3 disease together for the purposes of this comparison, cohort sizes remain unmatched due to the reduced frequency of complicated disease at presentation.

3.6 Can we better utilise existing clinical tools to optimise the triage of urgent referrals with suspected IBD and prompt earlier referral from primary care?

I have already touched upon the absence of a contemporary paper presenting symptom prevalence at IBD diagnosis in contrast to non-IBD diagnoses and alongside the primary screening tool used to identify IBD, FCP. Prospectively collected FCP levels and standardised clinical histories were modelled, as explored in the methods chapter. The aims were to:

- To identify the preferred FCP cut-offs that best predict IBD.
- To assess symptom frequency and discriminant ability in a cohort pre-selected by FCP.
- To identify symptom complexes which, when coupled with FCP results, carry the highest pre-test probability of IBD.

This process has been delivered pragmatically and therefore, whilst our objectives have been clear, we did not require patients to provide two FCP samples to progress through the pathway. This has resulted in various patient numbers contributing to each of the analyses presented in this section. An overview of this is given in ***Figure 3-7.***

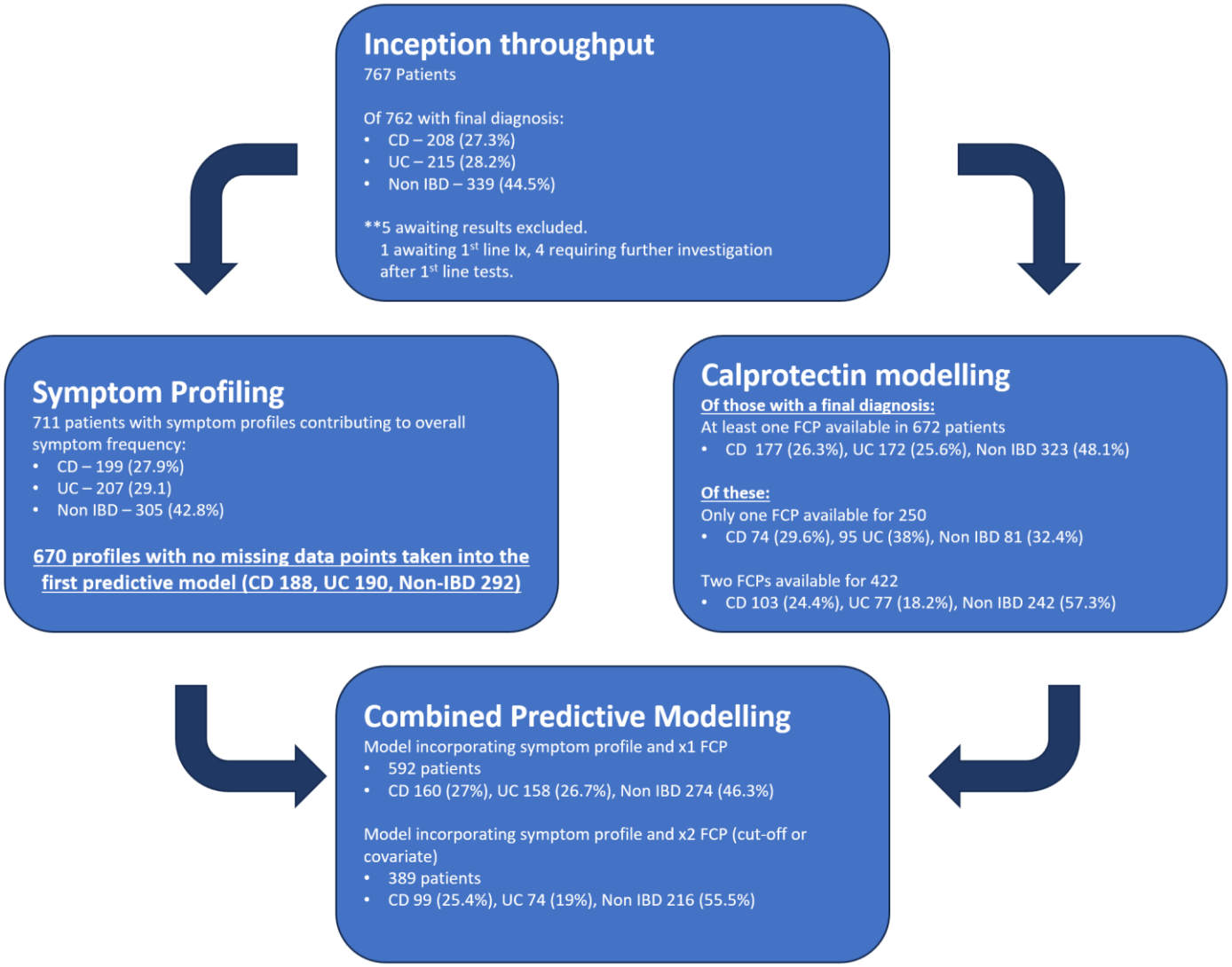


Figure 3 - 7: Total pathway throughput and subsequent patient numbers involved in the profiling and modelling undertaken using standard clinical datasets.

Overall pathway throughput totally 767 patients, with partial symptom profiles from 711 and complete symptom profiles from 670. Baseline and repeat FCP results were also obtained. Where modelling is presenting using both symptoms and FCP, there is drop off in the number of patients contributing. This fell to 389 patients who could be modelling on both symptoms and two FCP results.

3.6.1 FCP as a discriminator in IBD diagnosis – a question worth asking twice.

In our cohorts, all FCP testing was undertake using the Buhlmann fCAL® turbo test (Buhlmann laboratories, Basel). Of 672 patients seen via the pathway with at least one FCP result available, 581 (86%) had a result available at the time of initial referral. Only 258 (38%) had enteric infection excluded with a stool microscopy, culture and sensitivity sent at the same time. Ultimately, 422 patients had two FCP results available. However, the vast majority (337/422, 80%) of these were submitted in secondary care at the point of outpatient review, using the sample pot we had sent in the post. Consequently, the time between first and second FCP sample in our cohort was longer than previously suggested in the available literature (n=406 where time difference could be calculated, median 61d [IQR 63]).

Table 3-3 shows the difference in initial and repeat FCP, with the first result split by those who did and did not provide a second sample. The baseline cohorts were split in this way to attempt to identify if there were factors that inferred an increased likelihood of only one sample. Across all diagnoses, patient age was higher in the cohort providing one FCP (1FCP median 39 [IQR 25], 2FCP median 33 [IQR 18], Mann-Whitney U p<.001), whilst a higher proportion were male (55.7% vs 38.7%, X^2 p<.001). The only sub-diagnosis showing a significant difference in baseline FCP between those providing one or two samples was non-IBD, where patients with a higher index FCP were perhaps unsurprisingly more likely to provide a second sample.

Table 3 - 3: Differences in FCP at baseline compared between the cohorts providing one and two FCP results, with subsequent comparison of baseline and repeat FCP in those providing two, split by diagnostic subgroup.

	Baseline FCP ug/g (1 result only)*	**Baseline FCP ug/g (2 results only)***	**Baseline group comparison (within subgroup)**	**Repeat FCP ug/g (2 results only)**	**Repeated measure comparison (within subgroup)**
Crohn's Disease					
N=	N=74	N=103	Mann-Whitney U p=0.21	N=103	Wilcoxon rank p=0.14
Median (IQR)	664ug/g (1300)	915ug/g (1432)		592ug/g (1347)	
Ulcerative Colitis					
N=	N=95	N=77	Mann-Whitney U p=0.95	N=77	Wilcoxon rank p=0.09
Median (IQR)	1213ug/g (1510)	1293ug/g (1577)		1990ug/g (1742)	
Non-IBD					
N=	N=81	N=242	Mann-Whitney U p=0.002	N=242	Wilcoxon rank p<0.001
Median (IQR)	291ug/g (444)	391ug/g (641)		36ug/g (118)	

FCP = Faecal calprotectin, IQR = Interquartile Range

The distribution of these results is further explored in ***Figure 3-8.***

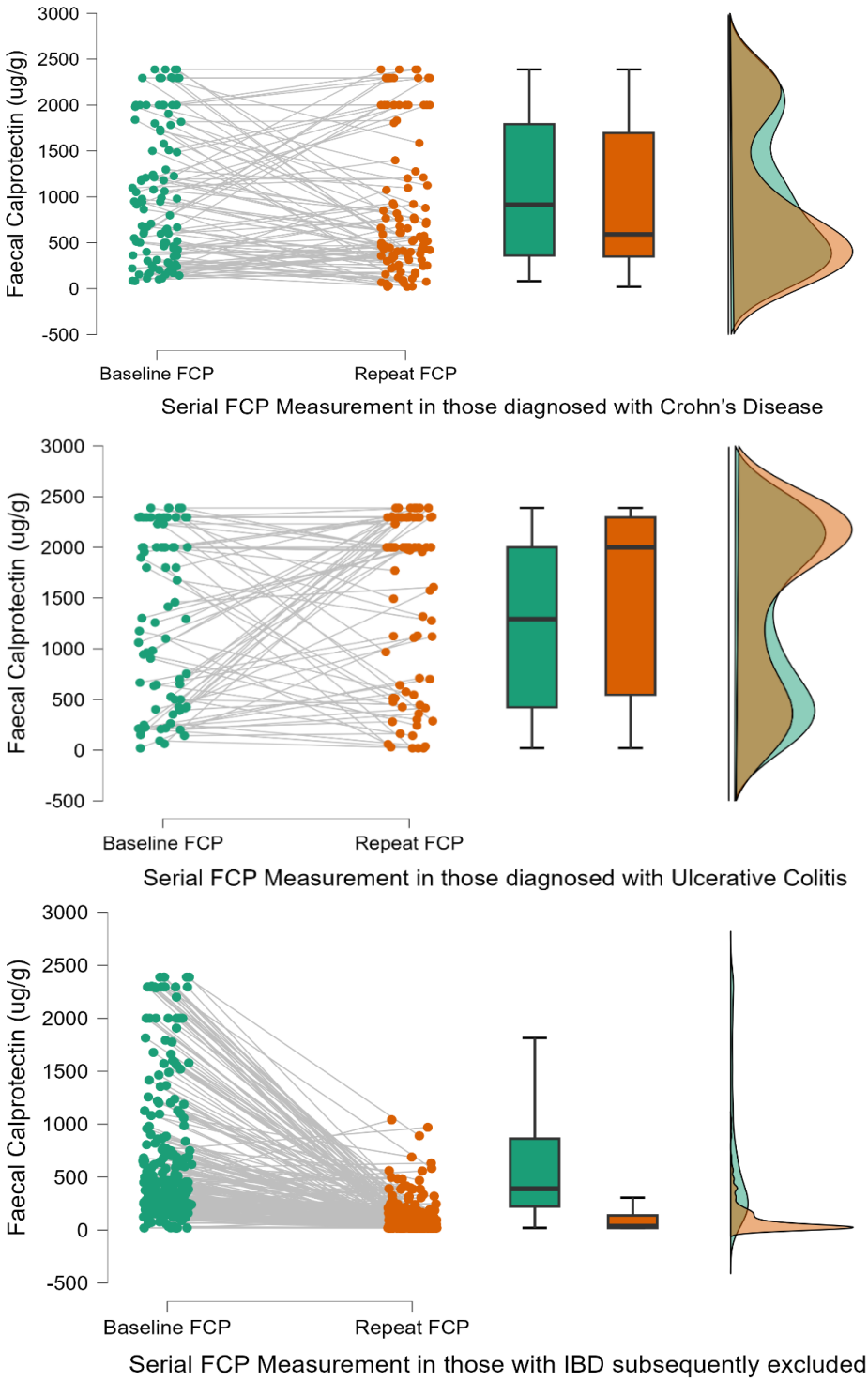


Figure 3 - 8: FCP at referral and at subsequent appointment, split by eventual diagnosis.

At baseline (n=672) and upon repeat (n=422), using Kruskal-Wallis(2) and Dunn pairwise test, there are significant differences between both CD (p_{holm}<0.001) and UC (p_{holm}<0.001) relative to non IBD. FCP was higher in UC than CD at baseline (p_{holm} 0.003) and repeat (p_{holm} 0.05). Amongst patients providing two samples, these plots demonstrate the difference in initial and repeat FCP when each subgroup is examined in isolation. Median FCP fell markedly in the non-IBD cohort, but not in either IBD subtype (UC n=77 Wilcoxon p=0.09, CD n=103 Wilcoxon p=0.14, non-IBD n=242 Wilcoxon p<.001). In fact, in the non-IBD cohort, the repeat FCP fell from significantly elevation to well within them at 36ug/g on repeat, highlighting the ability of repeated testing to prevent unnecessary invasive investigations.

Given the significant temporal changes in FCP, the overall predictive values and the performance of set cut-offs often suggested in clinical pathways was tested. In the first approach, all available first and second FCP results were plotted as receiver operator characteristic (ROC) curves. This is shown in ***Figure 3-9*** and highlights the substantial increase in predictive ability associated with the second FCP sample, albeit in cohorts that are unmatched in size.

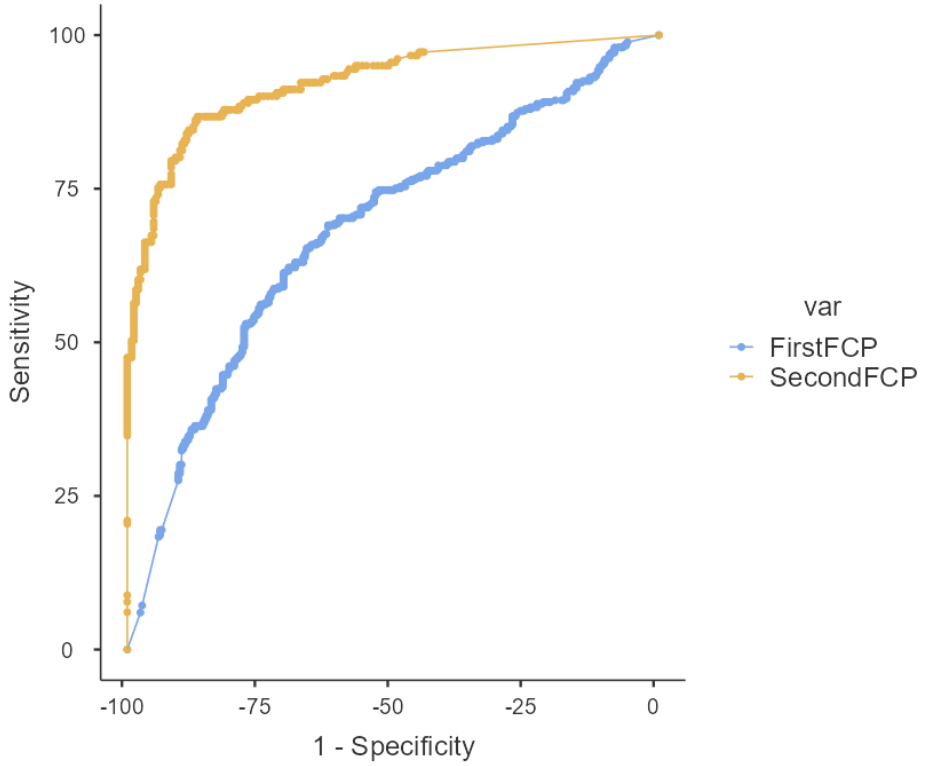


Figure 3 - 9: ROC curves for all 1st (n=672) and 2nd (n=422) FCP results predicting a diagnosis of 'IBD' vs 'non-IBD'.

For all first 1st, the area under the curve (AUC) was 0.683. This increased to 0.923 for the available 2nd FCP results. The optimal cut point, derived utilising Youden's index, was seen at a 2nd FCP value of 220ug/g (Youden's index 0.735).

When analysing set cut-offs, analyses were only undertaken in those who had two results available to ensure each result was matched and datasets comparable. As such, the ***Table 3-4*** below is derived only from the 422 patients with two sample results available prior to diagnosis / treatment.

Table 3 - 4: Real world performance of Faecal Calprotectin (Buhlmann) Cut-offs in a 'Rapid access' IBD Inception clinic

FCP cut-offs	Sens (%)	Spec (%)	PPV (%)	NPV (%)	AUC
>50ug/g	99.4	3.7	44	90	0.516
>100ug/g	97.2	6.2	44	75	0.517
>150ug/g	93.9	12.0	44	73	0.530
>200ug/g	89.5	24.0	47	75	0.567
>250ug/g	82.3	31.4	47	70	0.569
>50ug/g x2	94.4	56.7	62	93	0.755
>100ug/g x2	89.8	70.2	69	90	0.800
>150ug/g x2	84.8	78.2	74	87	0.815
>200ug/g x2	79.8	87.0	82	85	0.834
>250ug/g x2	75.3	89.5	84	83	0.824

Sens = Sensitivity, Spec = Specificity, PPV = Positive Predictive Value, NPV = Negative Predictive Value, AUC = Area Under the Curve

The strongest performance in terms of Area under the Curve (AUC) was seen with two FCPs above 200ug/g (AUC 0.834). In our dataset however, this cut off missed 36 patients who were subsequently found to have IBD during investigation. 16 of this

group had significant increases between the 1st and 2nd result (median 152.5ug/g baseline vs 380.5ug/g repeat) which still emphasised the possibility of an IBD diagnosis. However, this left 20 patients 20 where a diagnosis may have been missed without careful consideration. The issue of FCP in ileal disease was apparent as this group contained 13 with CD, of whom 9 had limited ileal disease. The rest had UC with limited proctitis in 6. Indeed, both ileal disease (1st FCP, L1 n=79 median FCP 400ug/g, L2 n=47 1403ug/g, L3 n=51 1052ug/g. Dunn pairwise test L1vsL2 p_{holm}<0.001, L1vsL3 p_{holm}<0.001) and proctitis (1st FCP, E1 n=56 median FCP 653.5ug/g, E2 n=59 1459ug/g, E3 n=57 1800ug/g. E1vsE2 p_{holm}0.001, E1vsE3 p_{holm}0.001) showed consistently lower FCP levels than CD with colonic involvement or more extensive UC respectively. This drop off in ileal disease has been previously explored. Whilst it has still been showing to correlate with both radiological and endoscopic ileal inflammation (Cerrillo et al., 2015; Jones et al., 2018), it is known to be less efficacious than in colonic Crohn's (Abej et al., 2016). Indeed, a recent literature review in this area suggested a lower threshold of 100ug/g for suggesting disease activity in those with established ileal disease on treatment (D'Amico et al., 2021).

In view of this, whilst a cut off >200ug/g x2 would seem a reasonable threshold to use for straight to test colonoscopy in those outside of traditional 'two-week wait' criteria, a lower threshold of >100ug/g x2 could be utilised to prompt clinic review and reduce false negatives. Certainly, in our data this still carried an AUC of 0.800 but increased the sensitivity for IBD from 79.8% to 89.8%.

3.6.2 The relationship between Faecal Calprotectin, disease severity and treatment outcomes

FCP is well established as a marked of disease activity in those with established IBD. In prospective studies in patients with UC, FCP has been shown to strongly correlate with both endoscopic and histological disease activity (Walsh et al., 2019). Another study across UC and CD compared FCP with traditional biochemical parameters such as white cell count (WCC) and C-Reactive Protein (CRP) and FCP to be a superior marker for histological disease activity (Kyle et al., 2020). Such is its role as a surrogate marker, FCP has been included as an intermediate treatment target in the recently updated STRIDE 2 guideline (Turner et al., 2021).

Using the larger cohort of patients associated with the first FCP result, patients who went on to require biologic therapy had a significantly higher Faecal Calprotectin at referral than those that did not. This was true across IBD (1508vs831ug/g, Mann-Whitney U 11000 p<0.001) and when looking at IBD subtypes ***(Figure 3-10)***.

Moreover, using a standardised approach (see methods), UC patients who were deemed not to have had a treatment response at 6 months had a significantly higher FCP at baseline that those that responded (n=86 2000vs982ug/g, Mann-Whitney U 290.5, p 0.03). This was not significant in CD (p=0.65). This difference was not seen at 12 months post diagnosis.

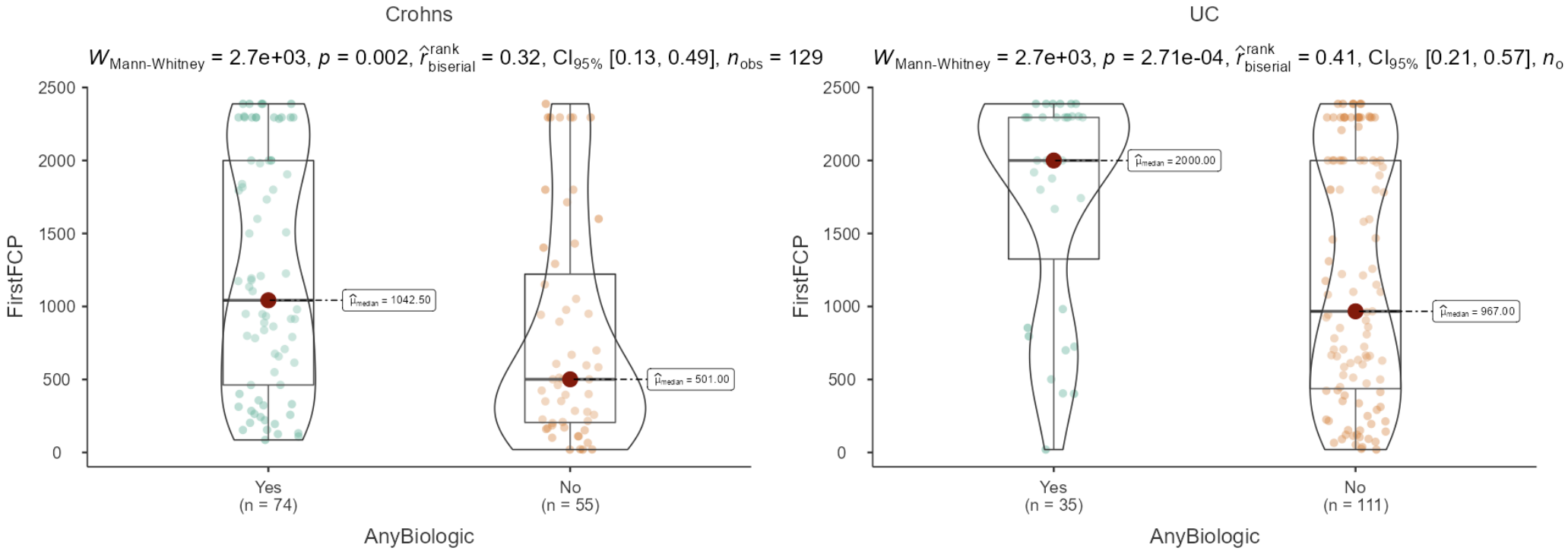


Figure 3 - 10: Baseline FCP in CD and UC, split but future need for biologic therapy

A higher FCP at baseline was associated with a higher likelihood of future biologic use in both CD (n=129, Mann-Whitney 2700, p=0.002) and UC (n=146, Mann-Whitney 2700, p=.001). This difference was greater in those with UC. This data includes a maximum follow up duration of 36 months.

3.6.3 Revisiting the clinical history: Presenting symptom profiles and diagnostic discriminators

Symptom profiles were prospectively collected from all patients across 13 standardised domains. These were available, as shown in ***Figure 3-7***, for 199 CD patients, 207 UC and 305 with non IBD. Any with missing data are included in the overall symptom frequencies but excluded from any modelling presented. Overall responses are shown in ***Table 3-5.***

The 5 most frequently reported symptoms in CD were abdominal pain (84%), looser stools (84%), fatigue (79%), faecal urgency (63%) and PR bleeding (any type, 55%). The most common 5 symptoms in UC were PR bleeding (any type, 94%), faecal urgency (82%), looser stools (81%), abdominal pain (68%) and fatigue (63%). The biggest differences between CD and UC were the increased prevalence of abdominal pain, fatigue, weight loss and joint pain in CD whilst rectal bleeding, urgency and mucous increased in UC. Whilst common in CD, abdominal pain was also highly prevalent in the non-IBD cohort). The largest differences seen across both IBD subtypes and non-IBD were in the increased frequency of weight loss and the passage of blood per rectum mixed in with stools in IBD.

Using these symptom frequencies, a regression model was developed to highlight these differences and home in on symptoms that are particularly helpful in separating CD from largely functional non-IBD diagnoses. This model, for each IBD subtype, is shown in ***Table 3-6***.

Table 3 - 5: Symptom Frequencies at presentation in CD, UC and grouped non-IBD diagnoses (n=711). Chi-squared p-value presented for global differences and variation between IBD subtypes.

	Crohn's (n = 199) % (n of responses +ve)	**UC (n = 207) % (n of responses +ve)**	**Non-IBD (n = 305) % (n of responses +ve)**	**X^2 p value**
Symptom duration >1m	97% (193/199)	92% (190/207)	89% (279/305)	Global: 0.007 CDvsUC: 0.005
Gastrointestinal symptoms				
Looser stools	84% (167/199)	81% (169/207)	74% (212/305)	Global: 0.01 CDvsUC: 0.53
Nocturnal BO	37% (72/194)	46% (91/197)	27% (81/303)	Global: <.001 CDvsUC: 0.06
Faecal Urgency	63% (126/199)	82% (170/207)	55% (167/305)	Global: <.001 CDvsUC: <.001
Faecal incontinence	10% (20/199)	12% (24/207)	7% (22/305)	Global: 0.22 CDvsUC: 0.61
Constipation	23% (45/199)	17% (35/207)	34% (104/305)	Global: <.001 CDvsUC: 0.16
PR Blood (any type)	55% (110/199)	94% (194/207)	49% (149/305)	Global: <.001 CDvsUC: <.001
Type of PR blood Anorectal Mixed None	 23% (45/199) 33% (65/199) 45% (89/199)	 44% (90/207) 50% (104/207) 6% (13/207)	 38% (117/305) 11% (32/305) 51% (156/305)	 Global: <.001 CDvsUC: <.001
PR Mucous	16% (32/198)	37% (77/206)	28% (86/304)	Global: <.001 CDvsUC: <.001
Abdominal pain	84% (168/199)	68% (141/206)	87% (266/305)	Global: <.001 CDvsUC: <.001
Extra-Intestinal Symptoms				
Joint pain	27% (54/197)	18% (36/203)	21% (65/305)	Global: 0.06 CDvsUC: 0.02
Fatigue	79% (155/196)	63% (126/201)	50% (151/300)	Global: <.001 CDvsUC: <.001
Weight loss	53% (104/198)	36% (75/207)	23% (69/305)	Global: <.001 CDvsUC: 0.001
Constitutional Symptoms	10% (19/198)	6% (13/206)	9% (27/305)	Global: 0.44 CDvsUC: 0.23
Mouth Ulcers	16% (31/197)	11% (23/204)	10% (31/304)	Global: 0.16 CDvsUC: 0.20
Risk factors				
Smoking status Current Ex Never	 21% (41/199) 18% (36/199) 61% (122/199)	 7% (14/204) 27% (55/204) 66% (135/204)	 20% (61/304) 16% (47/304) 64% (196/304)	 Global: <.001 CDvsUC <.001
Family History of IBD	26% (52/197)	15% (30/202)	13%(40/301)	Global: <.001 CDvsUC 0.005

Table 3 - 6: Predictive ability of presenting symptoms for IBD subtypes over non-IBD diagnoses across 670 patients.

Multivariate Model
Prediction of Crohn's, UC or Non-IBD
Model Fit: McFadden's PseudoR^2 0.253, Overall Model Test X^2 360 df 36 p<0.001.

	Crohn's – Non IBD				UC – Non IBD		
Predictor (Y-N)	**OR**	**95% CI**	**P**	**Predictor (Y-N)**	**OR**	**95% CI**	**P**
Model intercept	0.07	0.02 – 0.24	<.001	**Model intercept**	0.07	0.02 – 0.24	<.001
Symptom duration >1m	3.89	1.42 – 10.62	0.01	**Symptom duration >1m**	1.83	0.79 – 4.27	0.15
Looser stools	1.43	0.77 – 2.66	0.25	**Looser stools**	0.38	0.20 – 0.77	0.01
Constipation	0.78	0.47 – 1.28	0.32	**Constipation**	0.65	0.37 – 1.15	0.14
Nocturnal bowel opening	0.86	0.57 – 1.60	0.86	**Nocturnal bowel opening**	1.84	1.05 – 3.24	0.03
PR Bleeding:				**PR Bleeding:**			
Anorectal – None	0.99	0.60 – 1.65	0.98	**Anorectal – None**	11.12	5.41 – 22.84	<.001
Mixed – None	4.38	2.40 – 7.98	<.001	**Mixed – None**	33.68	15.47 – 73.33	<.001
Mucous	0.39	0.22 – 0.69	.001	**Mucous**	0.83	0.50 – 1.39	0.49
Faecal Urgency	0.84	0.50 – 1.42	0.51	**Faecal Urgency**	4.37	2.25 – 8.48	<.001
Incontinence	1.20	0.55 – 2.60	0.65	**Incontinence**	1.27	0.57 – 2.25	0.55
Weight loss	3.39	2.14 – 5.38	<.001	**Weight loss**	2.33	1.37 – 4.00	.002
Abdominal pain	0.51	0.27 – 0.95	0.03	**Abdominal pain**	0.22	0.12 – 0.40	<.001
Joint Pain	0.92	0.56 – 1.52	0.75	**Joint Pain**	0.72	0.40 – 1.28	0.26
Mouth Ulcers	1.13	0.60 – 2.12	0.72	**Mouth Ulcers**	0.88	0.42 – 1.84	0.74
Fatigue	2.94	1.77 – 4.87	<.001	**Fatigue**	1.63	0.96 – 2.78	0.07
Constitutional symptoms	0.80	0.38 – 1.71	0.56	**Constitutional symptoms**	0.57	0.24 – 1.41	0.23
Family History	2.05	1.21 – 3.49	<.001	**Family History**	0.81	0.43 – 1.52	0.51
Smoking status:				**Smoking status:**			
Current – Non	1.08	0.62 – 1.86	0.79	**Current – Non**	0.43	0.21 – 0.90	0.03
Ex – Non	1.21	0.69 – 2.13	0.50	**Ex – Non**	1.50	0.85 – 2.65	0.16

Mixed blood in the stool was the most discriminant symptom for both CD (OR 4.38; 95% CI 2.40–7.98) and UC (OR 33.68; 15.47–73.34). Weight loss (OR 3.39; 2.14–5.38), fatigue (OR 2.93; 1.77–4.87) and family history of IBD (OR 2.05; 1.20-3.49) also associated with an increased likelihood of CD over non-IBD diagnoses. With regards to UC, weight loss was again a significant predictor (OR 2.33; 1.37–3.96). Faecal urgency (OR 4.37; 2.25–8.45) and nocturnal bowel opening (OR 1.84; 1.05-3.24) were also associated with UC diagnoses).

The association between presenting symptoms and endoscopic severity at diagnosis was also briefly explored. A multiple linear regression model including all 13 symptom domains as factors to predict endoscopic severity (UC- UCEIS, CD – SESCD). For UC (n=86, model R^2 0.365, overall model F 2.91 p<0.002), the presence of nocturnal symptoms (t 2.71 p=0.01) and weight loss (t 2.53 p=0.01) were associated with a higher UCEIS score. In an identical model for CD (n=125 R^2 0.325, overall model F 3.79 p<.001) weight loss was the only significant predictor of increased SESCD (t=2.65 p=0.01).

3.6.4 Can the addition of key clinical symptoms improve the discriminant ability of FCP?

Clearly it is important to highlight the differences of CD given patients with this condition disproportionately represent those with a delayed diagnosis. However, any referral pathway and model used for entry to it, needs to be able to differentiate between IBD as a whole and non-IBD. To explore this, two binomial regression models were developed. Both used the same symptom indices. Model A modelled

FCP results as a factor using a ‘yes/no’ for two FCPs >200ug/g. Model B modelled first and second FCP as covariates. The outputs from both models are presented in ***Table 3-7.***

The indices from these models significantly out-perform the overall outputs generated by the same binomial model based on symptoms alone (McFadden’s PseudoR^2 0.278, Overall Model X^2 148 df 18 p<.001. Optimal cut-off value 0.43, sensitivity 0.78, specificity 0.77, AUC 0.837). With the inclusion of FCP, the AUC increased to 0.929 in Model A. In this model it was possible to demonstrate a pre-test probability of IBD of 92.5% (95% CI 74.6-98.1) in patients with two FCPs >200ug/g and mixed bleeding. This increased to 95.9% (95% CI 84.0-99.1) if weight loss is also present, or 95.6% (95% CI 83-99) if weight loss is substituted for fatigue. If bleeding is removed but weight loss and fatigue modelled together at the same FCP threshold, the probability remained high at 91.9% (95% CI 73-97.9). However, changing approach and modelling FCP values as covariates as in Model B improved the overall strength of the model but in terms of fit and predictive ability (AUC 0.947).

Table 3 - 7: Ability to predict IBD over non-IBD modelling clinical history + FCP as a covariate or factor, n=389).

Model A Set FCP threshold >200ug/g x2 McFadden's PseudoR^2 0.511 Overall Model X^2 311 df 19 p<0.001					**Model B** Baseline and repeat FCP as covariates McFadden's PseudoR^2 0.580 Overall Model X^2 305 df 20 p<0.001			
	IBD – Non IBD					**IBD – Non IBD**		
Predictor (Y-N)	**OR**	**95% CI**	**P**		**Predictor (Y-N)**	**OR**	**95% CI**	**P**
Model intercept	0.01	.002 – 0.09	<.001		Model intercept	0.01	.001 – 0.12	<.001
Symptom duration >1m	3.43	0.84 – 14.00	0.09		Symptom duration >1m	6.58	0.61 – 71.32	0.12
Looser stools	1.50	0.60 – 3.74	0.38		Looser stools	1.06	0.41 – 2.77	0.90
Constipation	0.70	0.33 – 1.51	0.36		Constipation	0.93	0.42 – 2.06	0.86
Nocturnal bowel opening	0.84	0.38 – 1.90	0.68		Nocturnal bowel opening	0.78	0.33 – 1.85	0.58
PR Bleeding:					PR Bleeding:			
Anorectal – None	3.01	1.37 – 6.61	0.01		Anorectal – None	2.54	1.11 – 5.80	0.03
Mixed – None	12.23	4.68 – 31.93	<.001		Mixed – None	8.09	2.79 – 23.51	<.001
Mucous	0.73	0.34 – 1.60	0.44		Mucous	0.46	0.18 – 1.14	0.09
Faecal Urgency	1.09	0.51 – 1.42	0.83		Faecal Urgency	1.25	0.55 – 2.84	0.60
Incontinence	1.02	030 – 3.47	0.97		Incontinence	1.57	0.47 –5.22	0.46
Weight loss	3.65	1.74 – 7.66	<.001		Weight loss	2.62	1.20 – 5.73	0.02
Abdominal pain	0.36	0.15 – 0.92	0.03		Abdominal pain	0.28	0.10 – 0.74	0.01
Joint Pain	0.86	0.39 – 1.89	0.71		Joint Pain	1.13	0.50 – 2.55	0.76
Mouth Ulcers	1.43	0.55 – 3.72	0.46		Mouth Ulcers	1.28	0.48 – 3.47	0.62
Fatigue	3.11	1.43 – 6.75	.004		Fatigue	3.04	1.32 – 7.00	0.01
Constitutional symptoms	0.33	0.08 – 1.42	0.13		Constitutional symptoms	0.15	0.02 – 1.40	0.10
Family History	1.62	0.67 – 3.96	0.29		Family History	1.39	0.56 –3.50	0.48
Smoking status:					Smoking status:			
Current – Non	0.84	0.34 – 2.08	0.71		Current – Non	1.23	0.49 – 3.06	0.66
Ex – Non	1.11	0.47 – 2.64	0.81		Ex – Non	0.98	0.37 – 2.59	0.97
Both FCP >200ug/g	27.09	13.73-53.47	<.001		Baseline and repeat FCPs modelled as covariates			
Overall predictive indices	Optimal cut-off value 0.40				**Overall predictive indices**	Optimal cut-off value 0.35		
	Sensitivity: 0.84					Sensitivity: 0.89		
	Specificity: 0.84					Specificity: 0.88		
	AUC: 0.929					AUC: 0.947		

3.6.5 How can this be applied for earlier diagnosis?

This study represents the first attempt to prospectively document presenting IBD symptoms and FCP level in a IBD inception cohort and interrogate the predictive capacity of individual symptoms in a cohort largely pre-selected because of FCP. The most common symptoms in IBD did not always separate patients from non-IBD diagnosis, with abdominal pain, for example, highly prevalent in the non-IBD cohort.

Of traditional FCP thresholds, 200ug/g on two occasions performed best statistically but missed some mild IBD presentations. A two-sample >100ug/g threshold to trigger outpatient clinic review with two samples above 200ug/g to progressing directly to endoscopy would miss fewer IBD cases. However, plotting FCP alone, a second FCP of 220ug/g carried a higher AUC then any two-sample threshold. Modelling FCP values with symptoms further increased AUC and improved the predictive capacity. Specific symptom complexes carried a very high pre-test probability of IBD when couple with elevated FCP. In an era where the availability of home FCP testing is increasing, symptom complexes and FCP values could feed into algorithms that allow self-referral to secondary care services. The provision of simple symptom questionnaires and home delivered FCP testing could reduce strain on primary care, allow rapid identification and prioritisation of those most likely to have IBD.

There are several limitations in our approach. At present, our modelling identifies discriminant factors but does not present a readymade pathway to apply at the point of referral. Furthermore, generalisability is limited by the acquisition of these symptom histories by a small pool of doctors in one trust. Self-reporting has not been

validated in this study. Regarding the modelling, it is acknowledged that this represents a relatively small cohort for this to be based upon, particularly when broken down to only those with two FCP results and a complete symptom profile (Riley et al, 2020). The pragmatic application of inclusion criteria for the pathway may also allow for methodological inconsistency and difficulties in reproducibility. The majority of our second FCP results were not submitted until secondary care review, increasing the time between samples beyond that typically recommended. Whilst the return of stool samples amongst patients is known to be hit and miss (Lecky et al., 2014), it is an inherent risk that allowing patients to pass through the pathway with one or two samples, those with more fulminant symptoms or other unknown factors may skew the composition of the one or two FCP cohorts. Certainly, a higher proportion of those providing one FCP (67.6%) went to be diagnosed with IBD, whilst this fell to 42.6% in those providing two FCPs. Attempts have been taken to mitigate this in the presentation of the data. Any pathway subsequently developed from this data would require large scale multicentre validation.

Through the success of our work, I have had the opportunity to join the trial management group for an ambitious approach to this problem being led by the IBD team in Exeter. They are in the process of developing a direct to patient faecal calprotectin pathway, integrated with faecal immunochemical testing. This will be supported by targeted advertising, online resources and community access to testing kits via pharmacies in addition to traditional GP access. After supporting the initial roll out of this process and contributing to the approach taken using our own experiences, I aim to be able to try to replicate this pathway as part of a second

phase study. As has been clear with many prior calprotectin pathways, what works in one centre may not be directly applicable to another with a different patient population and resource limitation. What works in Devon may not necessarily work in the centre of Birmingham and before we can consider suggesting a wider roll out of such an approach, we must demonstrate its versatility and potential for success in diverse environments.

3.7 Recognising the Mental Health Burden at IBD onset and validating a Patient Reported Outcome Measure (PROM) for this purpose

There is a high prevalence of mental health disorders (MHD) in those with IBD, particularly anxiety and depression (Hinnant et al., 2024). Since 2016, there have been two large meta-analyses on this topic. The first (Neuendorf et al., 2016) looked at 158371 participants in 171 studies. They found an overall prevalence of anxiety disorder at 21% and anxiety symptoms in 35%. Depressive disorder was found in 15% with depressive symptoms in 22%. The prevalence of these symptoms was higher in those with CD vs UC and higher in those with active disease over remission. A 2021 analysis (Barberio et al., 2021) used more robust measures to look at 77 studies encompassing 30118 patients. Findings were similar with anxiety symptoms in 32.1%, depression symptoms in 25.2% and higher odds of anxiety (OR 1.2, 95% CI 1.1-1.4) and depression symptoms (OR 1.2, 95% CI 1.1-1.4) in CD over UC. Risk factors for both anxiety and depression included female sex and active disease. A link has also been established between IBD and deliberate self-harm (Umar et al., 2022). The relationship between IBD and MHDs can be viewed as reciprocal, with concerns regarding the unpredictability of the disease and impacts

on daily life driving further stress, higher rates of MHDs and further exacerbations of IBD symptoms (Sauk et al., 2023). It has been demonstrated, amongst those with established disease, that targeted psychological interventions, where a need is identified, can have a positive impact on IBD associated outcomes (Eccles et al., 2021).

Determining the prevalence of MHDs at the first presentation of IBD has been attempted. However, these studies often define new diagnoses loosely and include established disease of several years standing (Engel at al., 2021). Nonetheless, the IBD standards (2023) make clear the need for psychological assessment in the initial management of patients newly diagnosed with IBD. A 2023 review focussing on the practicality of delivering psychological support in established IBD patients highlighted the time constraints placed upon outpatient clinics (Kok et al., 2023). The authors recommended the utilisation of psychometric questionnaires such as the Hospital Anxiety and Depression Scale (HADS). Time for this is often limited in new patient clinics so the option to utilise a tool that will contribute to assessments of disease activity, overall disability and psychological disease burden is appealing.

3.7.1 Introduction: The IBD Disk

The IBD Disk was developed in 2017 (Ghosh et al., 2017). It represents a shortened version of the IBD Disability Index (Peyrin-Biroulet et al., 2012). The full version of the disk is shown in ***Figure 3-11***. It should be noted that unlike the IBD Disability Index upon which it is based, the development of the IBD Disk followed a somewhat

abridged process. The Disability Index was developed as a multi-organisation cooperation between the International Organisation on IBD, the International Society of Physical and Rehabilitation Medicine, The International Classification of Functioning Disability and Health - Research Branch and two branches of the World Health Organization. Its development was based on four preparatory studies, a consensus conference and a subsequent operationalisation process (Peyrin-Biroulet et al., 2012). The IBD Disk on the other hand was developed using an iterative Delphi consensus based upon a steering committee of 3 gastroenterologists, the rankings of 30 gastroenterologists and nurses attending an industry funded (AbbVie) IBD training programme and the refinements of a 14-gastroenterologist working group chosen by AbbVie (Ghosh et al., 2017). Nonetheless, the Disk originates from a need to have a broad PROM that can be delivered quickly in a busy clinical setting and cover the breadth of impact that IBD can have. It has been validated as a measure for daily life burden in a large multicentre study (Tadbiri et al., 2021). Disability as measured by the disk has been shown to correlate with CRP and FCP, whilst it has been shown to reduce over time with decreasing disease activity (Le Berre et al., 2020). More recently, albeit on a smaller scale, multiple disk domains have been shown to correlate with disease activity in ileal CD, as measured by bowel wall thickness on abdominal ultrasound (Katsaros et al., 2023).

However, its utility as a measure at disease onset, particularly with regard with psychosocial disease burden, has not been validated. The HADS score is a long-established validated measure to screen for major depression and anxiety amongst

individuals with physical health problems (Bjelland et al., 2002, Wu et al., 2021). Accordingly, it is an attractive comparator for any putative new tool.

Our aims therefore for this evaluation of the disk were to:

- determine If baseline IBD disk score can not only measure disease associated disability, but also independently screen for mental health symptoms
- determine if baseline IBD disk score, obtained prior to IBD diagnosis, predicts outcomes during the first year of treatment. This included the need for treatment escalation, the presence of persistent disease activity and the need for inpatient admission.

IBD Disk

Questionnaire and scoring disk

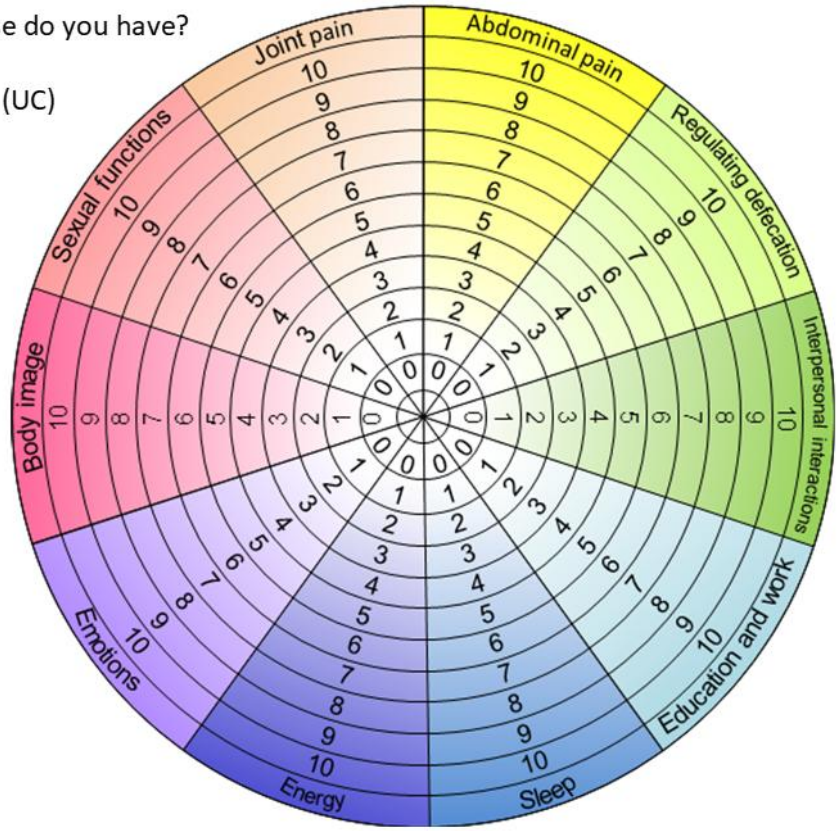
Please fill in this questionnaire and give to the doctor at your appointment. We will use this information to try and improve your care

Which bowel disease do you have?

☐ Crohn's disease

☐ Ulcerative colitis (UC)

☐ I don't know



In the last week, because of my Crohn's disease or ulcerative colitis:

Score each statement on a scale of 0 to 10 and circle your score on the colored disc

1 2 3 4 5 6 7 8 9 10

Absolutely disagree — Neither agree or disagree — Absolutely

Abdominal pain	I have aches or pains in my stomach or abdomen
Regulating defecation	I have had difficulty coordinating and managing defecation, including choosing and getting to an appropriate place for defecation and cleaning myself afterwards
Interpersonal interactions	I have had difficulty with personal relationships and/or difficulty participating in the community
Education and work	I have had difficulty with school or studying activities, and/or difficulty with work or household activities
Sleep	I have had difficulty sleeping, such as falling asleep, waking up frequently during the night or waking up too early in the morning
Energy	I have not felt rested and refreshed during the day, and have felt tired and without energy
Emotions	I have felt sad, low or depressed, and/or worried or anxious
Body image	I have not liked the way my body or body parts look
Sexual functions	I have had difficulty with the mental and/or physical aspects of sex
Joint pain	I have had pains in the joints of my body

Figure 3 - 11: The domains scored in the IBD Disk (Adapted from Ghosh et al., 2017).

The IBD disk is a measure composed of 10 questions, covering domains ranging from pain, faecal urgency *('regulating defecation')* to body Image and mood. Patients are asked to give an agreement score out of 10 for each statement, relating specifically to past week. A score of 10 indicates strong agreement and these scores are circled on a concentric grid. In its intended form, individual domain scores should be tracked against each other over time, though a total score can also be generated as a surrogate marker of disease activity and overall disability.

3.7.2 Methods

Between February 2021 and February 2024, patients attending outpatient clinics with a suspected but not confirmed diagnosis of inflammatory bowel disease were asked to complete an IBD disk score during clinic attendance. After interim analysis of disk data, a subgroup of patients was asked to complete a simultaneous HADS score to validate patterns emerging from earlier disk scores. These measures were collected prospectively alongside traditional clinical and biochemical indices and longitudinal treatment outcomes. Repeat scores were taken at follow up attendances alongside further clinical parameters. At each visit, current treatments were recorded, and an assessment of disease activity was undertaken (see methods section 2.2.2). Pre-existing mental health diagnoses were obtained from clinical history taking and GP coded diagnoses. Patients at follow up visits were stratified as either having ‘Active’ or ‘Inactive’ disease. Non-parametric tests were undertaken for all disk related analyses as both the total disk score (Shapiro-Wilk W 0.987 p=0.008) and each individual domain score did not follow a normal distribution. ROC curves were generated using the same Jamovi plug-ins as reported for FCP (Friesen et al., 2019, Theile et al., 2019).

3.7.3 Results

3.7.3.1 Defining the cohorts

IBD disk scores were collected from 305 patients at their first outpatient appointment, prior to diagnosis. 157 completed a simultaneous HADS score. Follow up IBD disk scores were collected at follow-up, post treatment visits from 82 IBD patients (99 overall), of whom 37 (49 overall) completed a paired post-treatment HADS score. The cohort providing baseline and follow up samples was predominantly IBD given the majority of those in the non IBD cohort, were discharged. To give clarity about the cohorts contributing to each sub analysis, a breakdown of each is given in ***Table 3-8*** below.

Table 3 - 8: Basic cohort descriptors for patients completing a IBD Disk or HADS

Total IBD disk visit 1 cohort				
	Crohn's	**UC**	**Non-IBD**	**Global test**
N	95	82	128	
Age median (IQR)	28 (14.5)	33.5 (16)	35 (17.5)	F = 6.09 p=0.048[1]
Sex (% male)	41%	57%	42%	X^2 5.85 p=0.054[2]
Baseline FCP, median (IQR), ug/g	791 (1266)	1597 (1540)	417 (565)	F = 41.16 p<.001[1]
Disease extent / location / non-IBD type (%)	Ileal: 47 (49) Colonic: 19 (20) Ileocolonic: 29 (31)	Proctitis: 22 (27) Left sided: 26 (32) Extensive: 34 (41)	Transient illness: 19 (15) Functional: 63 (49) Other GI: 46 (36)	
Pre-existing MHD (% yes)	21%	10%	31%	X^2 13.45 p=0.001[2]
Current antidepressants (% yes)	16%	6%	24%	X^2 11.87 p=0.003[2]
Current antipsychotics (% yes)	3%	0%	2%	X^2 2.43 p=0.296[2]
Cohort providing a paired HADS at visit 1				
N	43	41	73	
Age median (IQR)	28 (11.5)	32 (14)	33 (17.25)	F = 5.56 p=0.062[1]
Sex (% male)	37%	54%	33%	X^2 4.66 p=0.097[2]
Baseline FCP, median (IQR), ug/g	720 (1202)	1919 (1850)	377 (596)	F = 17.46 p<.001[1]
Disease extent / location / non-IBD type (%)	Ileal: 22 (51) Colonic: 10 (23) Ileocolonic: 11 (26)	Proctitis: 13 (32) Left sided: 13 (32) Extensive: 15 (37)	Transient illness: 12 (16) Functional: 30 (41) Other GI: 31 (42)	
Pre-existing MHD (% yes)	26%	12%	29%	X^2 4.14 p=0.126[2]
Current antidepressants (% yes)	21%	12%	19%	X^2 1.26 p=0.53[2]
Current antipsychotics (% yes)	5%	0%	4%	X^2 1.85 p=0.39[2]
Cohort providing a repeat IBD Disk				
N	49	33	17	
Age median (IQR)	27 (12)	33 (11)	35 (25)	F = 8.22 p=0.016[1]
Sex (% male)	40%	60%	33%	X^2 7.14 p=0.028[2]
Baseline FCP, median (IQR), ug/g	720.5 (852.75)	1949 (1338)	334 (736)	F = 17.91 p<.001[1]
Disease extent / location / non-IBD type (%)	Ileal: 23 (47) Colonic: 10 (20) Ileocolonic: 16 (33)	Proctitis: 5 (15) Left sided: 14 (42) Extensive: 14 (42)	Transient illness: 4 (24) Functional: 7 (41) Other GI: 6 (35)	
Pre-existing MHD (% yes)	24.5%	12%	47%	X^2 7.46 p=0.024[2]
Current antidepressants (% yes)	16%	6%	41%	X^2 9.78 p=0.008[2]
Current antipsychotics (% yes)	4%	0%	6%	X^2 1.69 p=0.430[2]
Visit 2 FCP median (IQR), ug/g	218 (644)	211.5 (272.75)	NA	F = 0.60 p=0.438[1]
Duration between visits (days)	114 (124)	118 (119)	131 (73.25)	F = 0.71 p=0.700[1]

[1] Kruskal-Wallis test [2] Chi-squared test

MHD = Mental Health Disorder

Both CD and non-IBD diagnoses are characterised by a significantly higher proportion of pre-existing mental health diseases than those with UC. This is particularly marked in the cohort in whom IBD was subsequently excluded. A total of 81 existing mental health diagnoses were identified with some individuals contributing multiple conditions. Of the total diagnosis, 58 (71.6%) were one of depression (25), anxiety (13) or a mixed anxiety and depressive disorder (20). The frequency of these three diagnoses, split by diagnostic subgroup, is shown in ***Figure 3-12.*** The most frequently utilised anti-depressants at presentation were Sertraline (18 [34% of all antidepressants]) followed by Citalopram (14 [26%]).

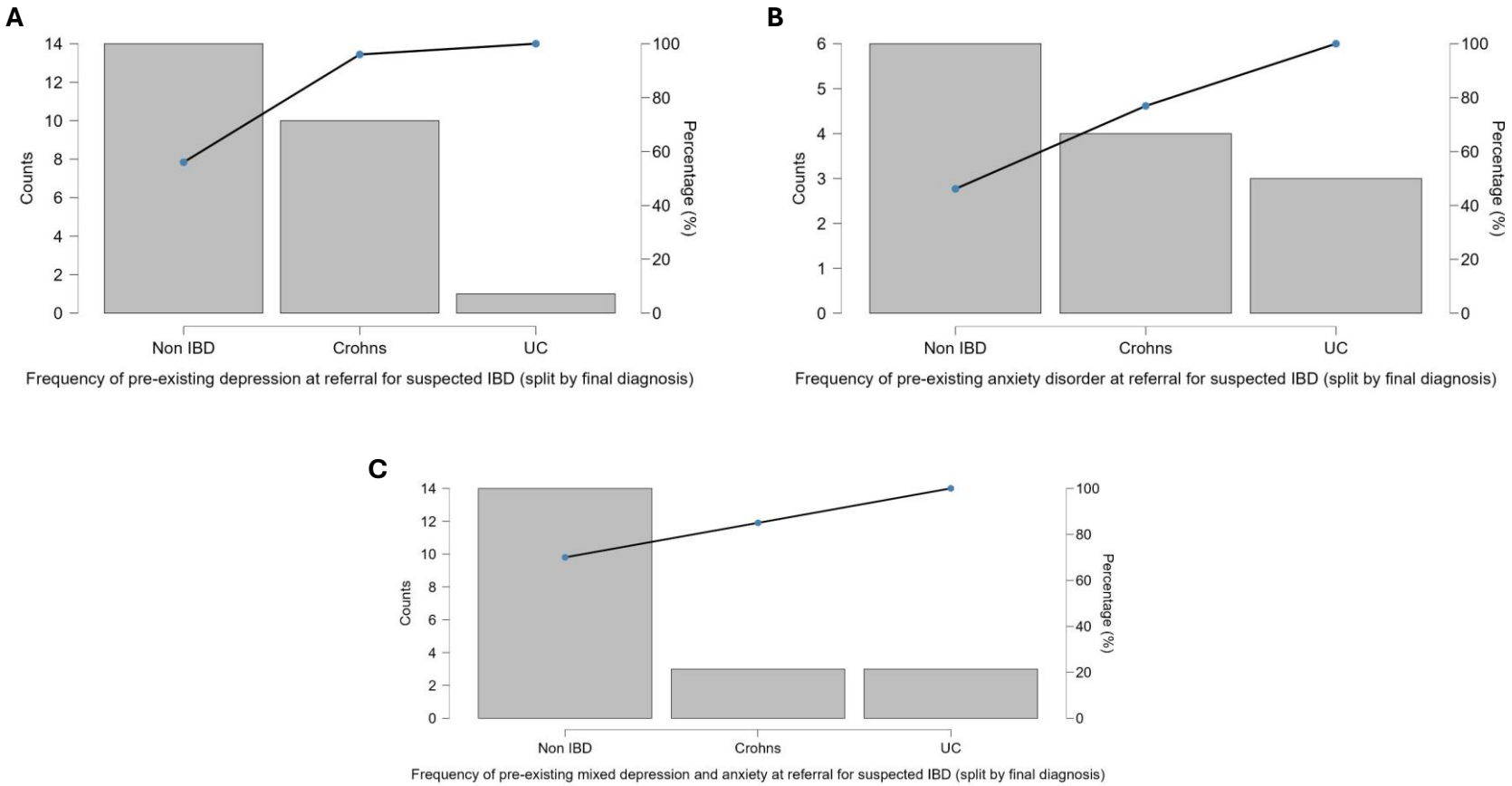


Figure 3 - 12: Pareto plots showing frequency of depression, anxiety or a mixed diagnosis in patients referred with suspected IBD, split by final diagnosis.

A. Pareto **plot A** demonstrates the frequency of depression in individuals referred. Of 25 with pre-existing diagnoses, 14 (56%) were from the 'non-IBD' group, with 10 (40%) subsequently diagnosed with Crohn's.
B. **Plot B** shows the more even spread amongst those with isolated anxiety disorder, though the largest proportion, 6 (46%), still arises from the non IBD group.
C. **Plot C** shows the distribution of those with a mixed anxiety and depressive disorder. 14 (70%) of those with this pre-existing diagnosis subsequently had IBD excluded.

3.7.3.2 Quantifying disability and psychological disease burden at baseline

The overall IBD disk score was highest in those who were subsequently diagnosed with CD (median 56.5, IQR 29). This value was significantly higher than that observed in Ulcerative colitis (median 44.5, IQR 36.5). Interestingly, the total disk score seen in patients where IBD was subsequently excluded was significantly higher than in UC (median 54.5, IQR 31.25. Kruskal-Wallis(2) 12.57 p=0.002, Dunn test CDvsUC p=0.003, Non-IBDvsUC p=0.005). The individual scores for each domain are presented, split by diagnosis, in ***Table 3-9.***

Two major patterns emerge from the data. Firstly, there are high levels of disability in those in whom IBD is subsequently excluded. This is in part explained by the significantly higher prevalence of existing mental health disorders and antidepressant use in this cohort. It is noteworthy that a pre-existing mental health diagnosis results in a significantly higher baseline IBD disk score in the Non-IBD cohort ('MH no' = median 51 'MH yes' = median 67. Mann-Whitney 1054.5 p<.001) and IBD cohort 'MH no' = median 49, 'MH yes' = median 58.5. Mann-Whitney 1572 p=0.039).

Table 3 - 9: *Median (interquartile range)* IBD disk domain scores (entire cohort) at baseline, split by final diagnosis, with the HADS scores from the relevant subgroup at the end of the table.

Domain	Crohn's	UC	Non IBD	Global test (Kruskal-Wallis)
Disk Domains (n=305)				
Abdominal Pain	9 (4)	5 (6)	8 (5.25)	p<.001
Regulating Defecation	5 (6.5)	4.5 (7)	4 (6)	p=0.94
Interpersonal Interactions	3 (6)	2 (4)	3.5 (7)	p=0.11
Education and Work	6 (6)	4 (6.75)	5 (7)	p=0.24
Sleep	7 (5)	5.5 (7)	6 (6)	p=0.12
Energy	9 (3)	8 (4)	8 (4)	p=0.003
Emotions	7 (4)	5 (5)	7 (3)	p=0.004
Body Image	6 (6)	3.5 (5)	6 (5.25)	p<.001
Sexual Functions	2 (7)	1.5 (5.75)	3 (6)	p=0.21
Joint Pain	5 (7)	2 (5)	6 (7)	p<.001
HADS Domains (n=157)				
Anxiety	8 (7.5)	7 (6)	10 (7)	p=0.02
Depression	6 (6.5)	4 (7)	7 (5)	p=0.02

Secondly, looking at IBD in isolation, another pattern emerges, with significantly increased disease burden at CD onset relative to UC. This is apparent over multiple domains utilising a post-hoc Dunn test for pairwise comparisons. CD demonstrated significantly higher agreement scores for the following domains : Abdominal pain (p_{holm} <.001), Energy (p_{holm}<.001), Emotions (p_{holm} 0.022) and body image (p_{holm} 0.003) than UC. The only significant difference CD and Non-IBD is seen in the fatigue domain with increased levels of fatigue in CD (p_{holm} 0.008). The differences across the fatigue domain are shown in ***Figure 3-13***.

Amongst IBD patients, the presence of a pre-existing mental health diagnosis did not result in a longer symptom duration at first review (No MHD median 6 months, MHD median 7.5 months, Mann-Whitney p=0.65). However, a longer symptom duration positively correlated with overall disk score (Spearman's r_2 = 0.195 p=0.01). Looking at individual disk domains, IBD patients with an 'Emotions' domain score of ≥7 had a significantly longer symptom duration that those that did not (n= 168, median 5 vs 11 months, Mann-Whitney p=0.004). This is even more striking when CD is looked at in isolation (n=91, median 6 vs 15 months, Mann-Whitney 1351 p=0.027). This pattern in CD follows a trend, outside of statistical significance, for an increased incidence of stricturing and penetrating complications at diagnosis in those with a longer symptom duration (Montreal B1 n=78 median=8.5 months, B2/B3 n=15 median=24 months, Mann-Whitney p=0.06).

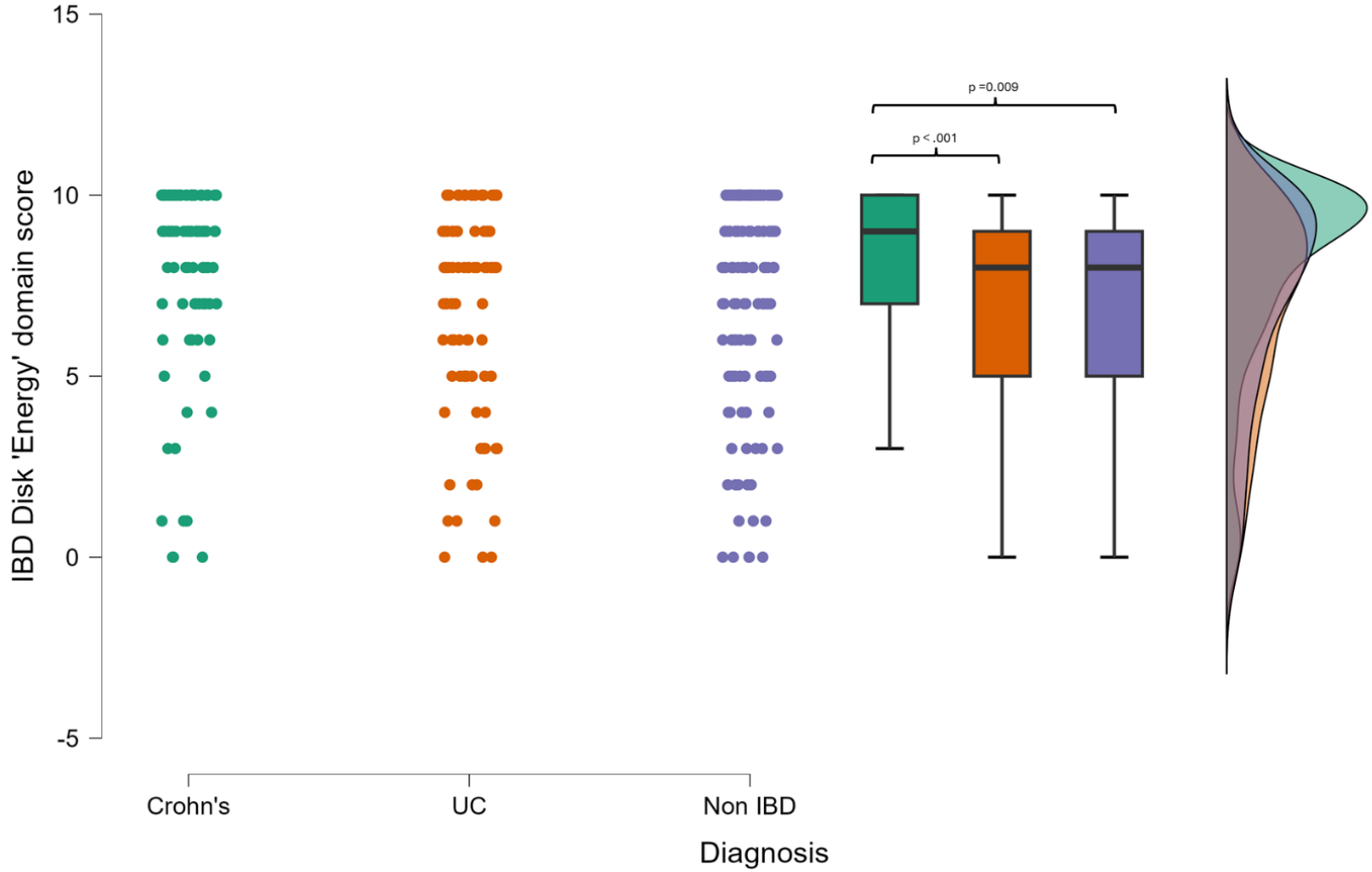


Figure 3 - 13: Increased Fatigue at presentation in CD

Agreement scores in response to the statement *'I have not felt rested and refreshed during the day, and have felt tired and without energy'* were significantly higher in those with CD than in those with UC or Non IBD diagnoses (n=304, Kruskal-Wallis[2] = 15.99 p<.001, Dunn test CD vs UC p<.001, CD vs Non IBD p=0.009).

3.7.3.3 Using the HADS to validate the IBD disk as a screening tool for significant psychological symptoms at IBD onset

Across all patients completing an IBD disk and a HADS score, strong correlations were seen between individual disk domains and HADS scores for both depression and anxiety. Given our objective is to validate the disk only at IBD onset, all subsequent analyses are presented just for those with IBD. ***Figure 3-14*** highlights the domains with the strongest association with HADS anxiety and depression during the baseline visit. For both scores, the *'Emotions'* domain outperformed all others.

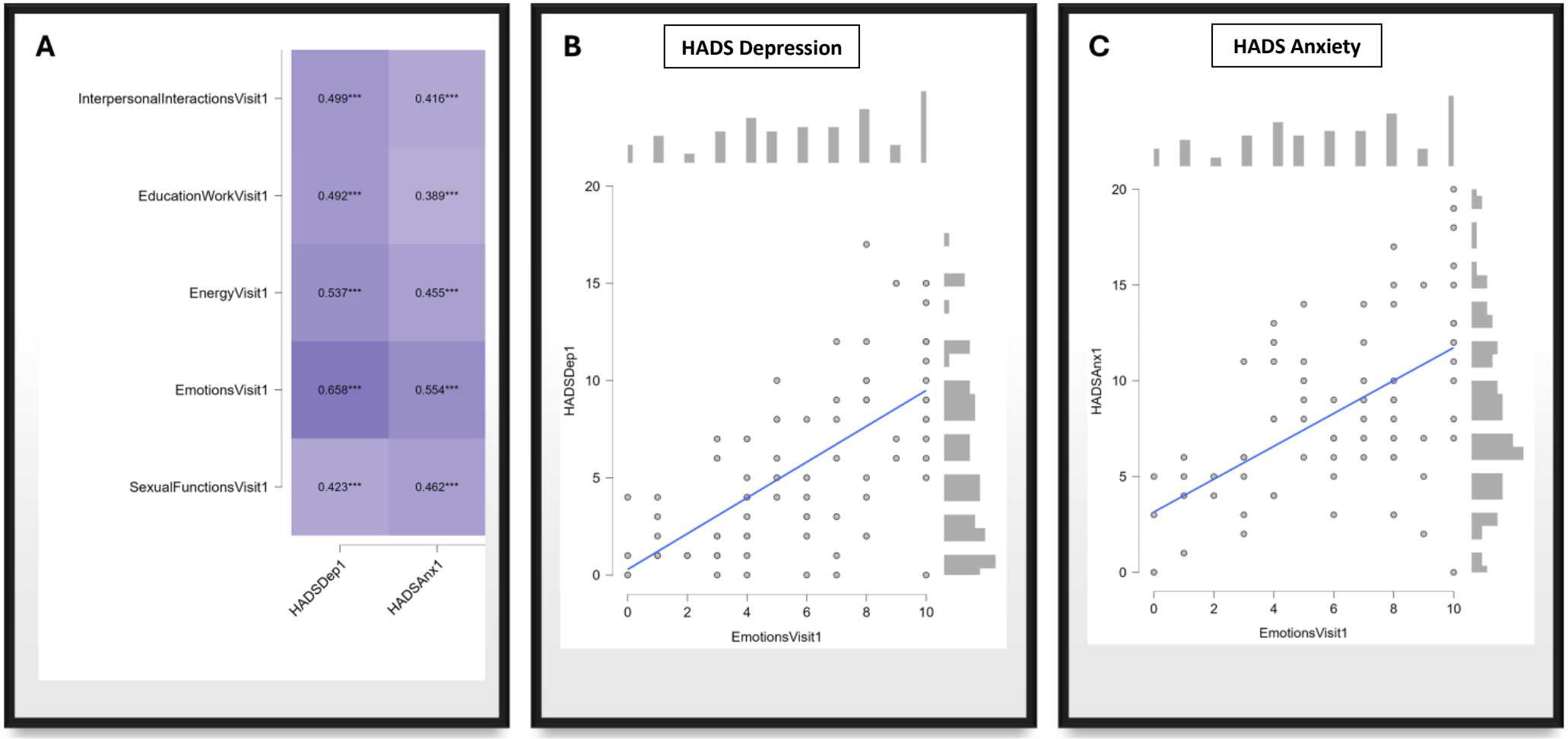


Figure 3 - 14: Heatmap of Spearman's rank coefficients for correlation between individual disk domains and HADS anxiety and depression scores amongst 84 patients presenting with IBD (43 CD, 41 UC). Individual scatter plots are shown for the *'Emotions'* domain.

A. This heatmap shows the top 5 IBD disk domains regarding correlation (Spearman's Rho) with HADS anxiety and depression. This analysis includes 84 IBD patients, 43 with CD and 41 UC. The strongest across both domains was the *'Emotions'* domain (HADS depression r_s 0.658 p<.001, HADS anxiety r_s 0.554 p<.001).
B. An individual scatter plot of paired HADS depression and IBD disk *'Emotions'* scores, with a histogram of score frequency around the outside of the plot.
C. An individual scatter plot of paired HADS anxiety and IBD disk *'Emotions'* scores, with a histogram of score frequency around the outside of the plot.

Even post treatment, during the second visit, disk '*Emotions'* scores remained most strongly associated with HADS Depression scores (n=36 r_s = 0.579 p<.001). As a next step, the *'Emotions'* domain was then utilised to find an appropriate cut off that would reliably detect those with at least moderate HADS determined symptoms of depression and anxiety (score ≥11 for each). Overall, the *'Emotions'* domain scores when plotted on a receiver operating characteristic (ROC) curve carried an area under the curve (AUC) of 0.875 for the HADS depression score cut-off. Statistically, the optimal cut point was a score of ≥8 (Sensitivity, 91.67%, specificity 70.83%, Youden's index 0.625), though a score of ≥7 capture all of those with significant depression scores (Sensitivity 100%, specificity 61.1%, Youden's index 0.611). For HADS anxiety the accuracy of the *'Emotions'* domain was lower, with an overall AUC of 0.754. The optimal cut-point statistically was an *'Emotions'* score of 10 (Sensitivity 48%, specificity 93.2%, Youden's index 0.412) though a lower threshold of ≥7 again carried greater clinical relevance as a screening tool (Sensitivity 72%, specificity 62.7%, Youden's index 0.347). The individual ROC plots of this data are shown in ***Figure 3-15.***

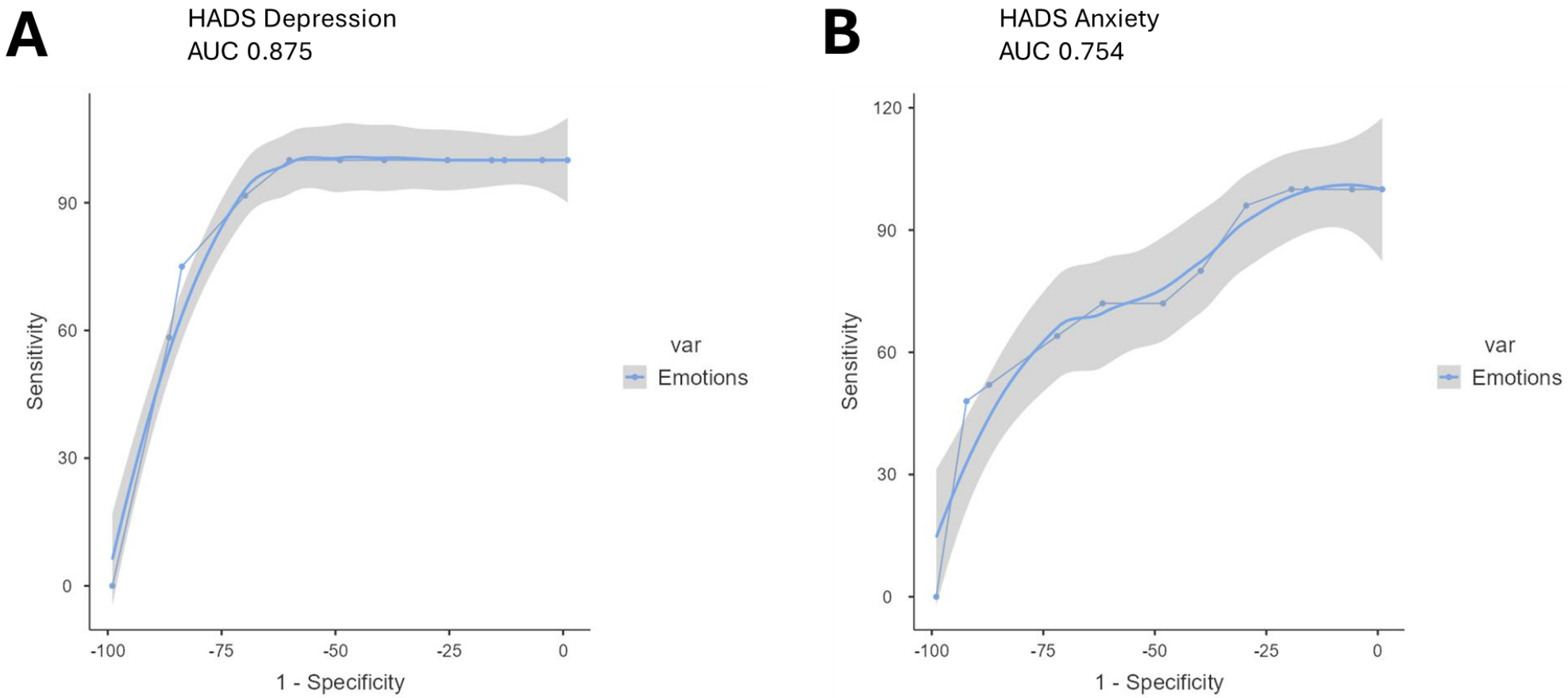


Figure 3 - 15: Receiver Operating Characteristic Curves of the ability of the IBD disk '*Emotions'* domain to identify HADS depression and anxiety scores of moderate severity (≥11) amongst 84 patients at first presentation of Inflammatory Bowel Disease.

Overall, the '*Emotions'* domain had an AUC of 0.875 for HADS depression scores ≥11, and 0.754 for HADS anxiety scores ≥11. For HADS depression, an *'Emotions'* domain score ≥8 had the highest Youden's index (0.625) but in a screening situation, a cut off ≥7 identified all individuals meeting the HADS cut off whilst retaining a high Youden's index (Sensitivity 100%, specificity 61.11%, Youden's index 0.611). The *'Emotions'* domain performed less well at pre-specified cut offs for HADS anxiety. Here the optimal cut-off statistically was 10 (Youden's index 0.412) but again a score of ≥7 would be favoured as it missed far few patients meeting the cut-off (Youden's index 0.347 but sensitivity 72%, specificity 62.71).

3.7.3.4 Disease activity is the key driver of ongoing disability and psychological disease burden after diagnosis

Once the diagnosis has been confirmed and treatment initiated, the burden of symptoms for patients attending their first follow up appointment was then quantified. Across the cohort, this appointment took placed a median of 117 (IQR 126.25) days after the first appointment.

In both CD and UC, the key driver of difference in the IBD disk score at the second visit is disease activity. Whilst the presence of a pre-existing mental health diagnosis resulted in a higher IBD disk score amongst IBD patients at baseline, this difference is not present at visit 2 ('MH no' = median 41 'MH yes' = median 50. Mann-Whitney 440 p=0.306). Furthermore, there is no proportional difference in patients reaching an inactive disease state by visit 2 when stratified by the presence of absence of a mental health diagnosis (X^2 0.047 p=0.828).

However, when patients are stratified by whether they have reached an inactive disease state at visit 2, a clear pattern emerges. Those with active disease at visit 2 carry a far higher overall disk score in addition to scores across multiple domains (V2 IBD Disk active = 55 [median], V2 IBD disk inactive = 27. Mann-Whitney 1370 p<.001). This is true in both CD (***Figure 3-16***) and UC (***Figure 3-17***).

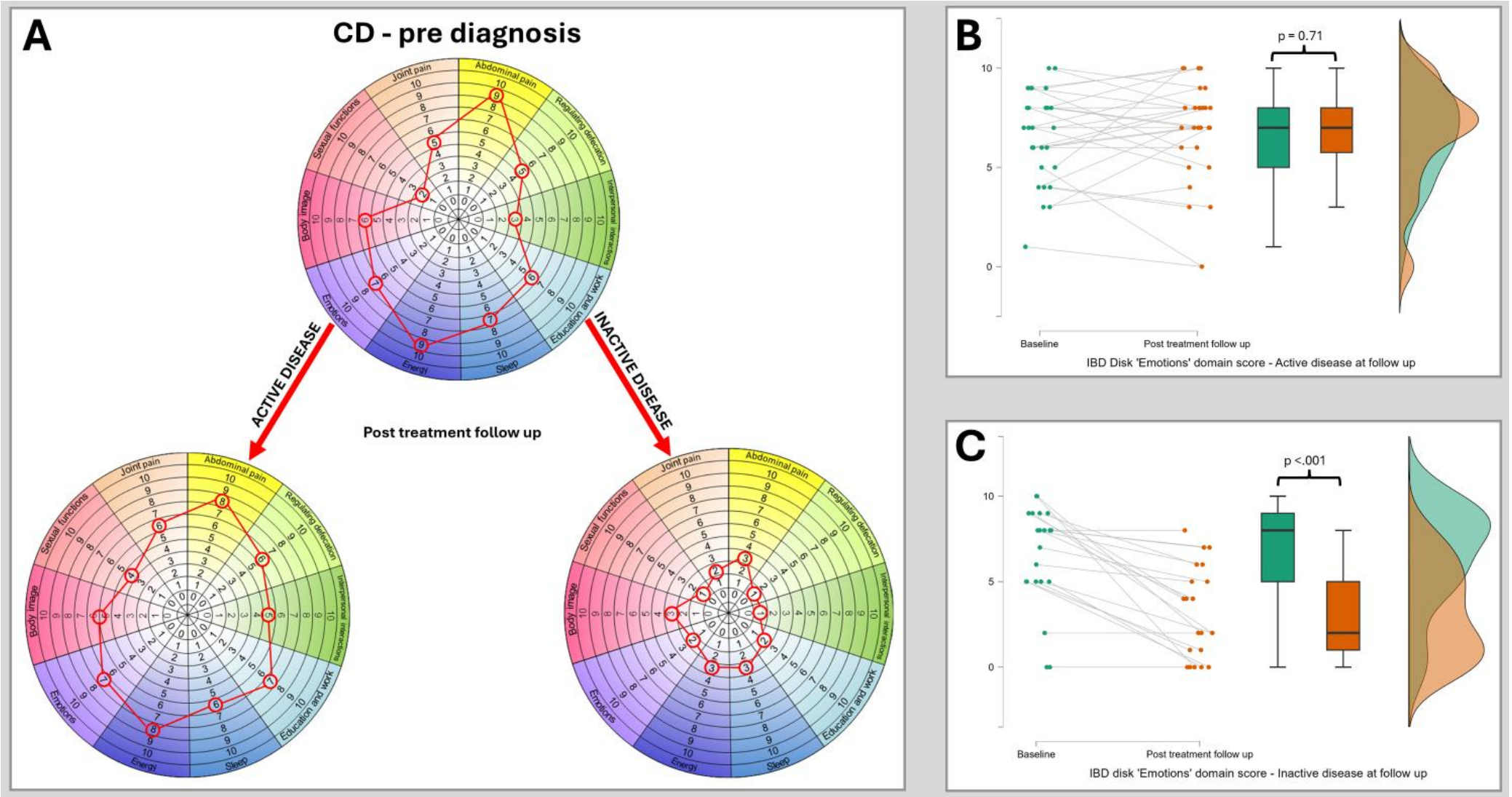


Figure 3 - 16: Median IBD disk scores for each domain in Crohn's disease at baseline and then at post treatment follow-up, stratified according to disease state.

A. Panel A plots median IBD disk scores at baseline and post treatment follow up for 49 patients with CD, of whom 28 had active disease at follow up and 21 were inactive. Scores are plotted on the grid as would be aim when interpreting these on an individual patient basis in clinc. The only significant reduction identified in the 'active' disease group was in the 'Energy' domain (Pre diagnosis median 9, post diagnosis median 8, Wilcoxon 170 p=0.015). All observed differences in the 'Inactive' disease group reached statistical significance.

B. Panel B shows a raincloud plot of IBD disk 'Emotions' domain scores at baseline and at first follow up in those with persistent disease activity. There is no significant difference observed (Pre diagnosis median 7, post diagnosis median 7, Wilcoxon 148.5 p=0.71).

C. Panel C shows a raincloud plot of IBD disk 'Emotions' domain scores at baseline and at first follow up in those who reached an inactive disease state at this time point. A highly significant reduction is observed in this cohort (Pre diagnosis median 7, post diagnosis median 2, Wilcoxon 153 p<.001).

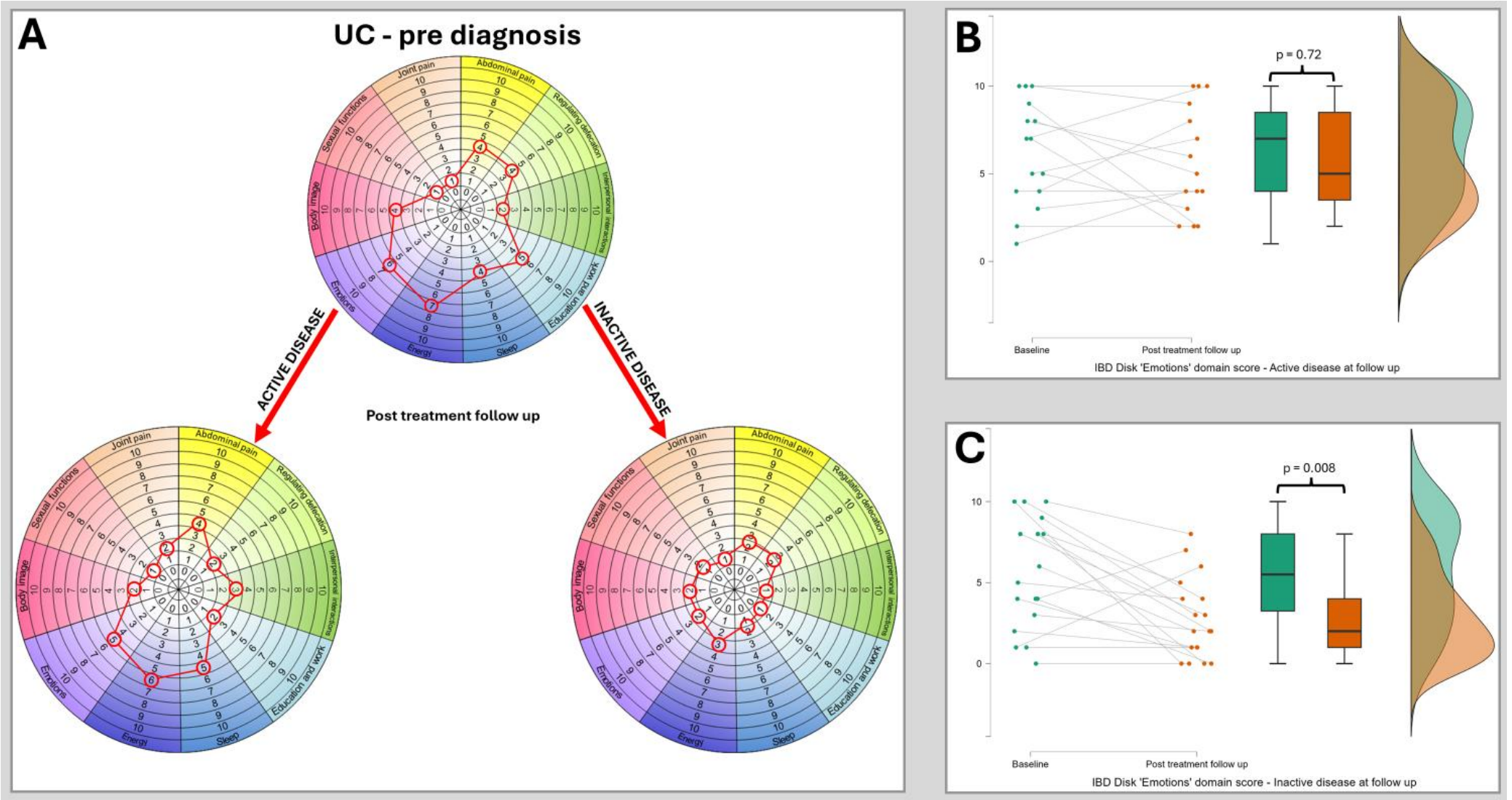


Figure 3 - 17: Median IBD disk scores for each domain in UC at baseline and then at post treatment follow up, stratified according to disease state

A. Panel A plots median IBD disk scores at baseline and post treatment follow up for 33 patients with UC, of whom 15 had active disease at follow up and 18 were inactive. Scores are plotted on the grid as would be aim when interpreting these on an individual patient basis in clinc. The only significant reduction identified in the 'active' disease group was again in the 'Energy' domain (Pre diagnosis median 7, post diagnosis median 6, Wilcoxon 56 p=0.044). In the 'Inactive' disease group differences in the *'Emotions', 'Abdominal pain', 'Regulated defecation', 'Education & work', 'Sleep' and 'Body image'* displayed statistically significant differences.
B. Panel B shows a raincloud plot of IBD disk 'Emotions' domain scores at baseline and at first follow up in those with persistent disease activity. There is no significant difference observed (Pre diagnosis median 7, post diagnosis median 7, Wilcoxon 37.5 p=0.72).
C. Panel C shows a raincloud plot of IBD disk 'Emotions' domain scores at baseline and at first follow up in those who reached an inactive disease state at this time point. A highly significant reduction is observed in this cohort (Pre diagnosis median 7, post diagnosis median 2, Wilcoxon 119.5 p=0.008).

3.7.3.5 Can baseline IBD-disk determined disability help predict outcomes in the first 12 months after IBD diagnosis?

A total of 156 IBD patients (85 CD, 71 UC) had complete 12-month outcomes available. First the impact of pre-existing MHD was quantified. CD patients with a pre-existing MHD received double the number of courses of oral corticosteroids during their first year of treatment (MHD no median=1 vs MHD yes median=2, Mann-Whitney 1084 p=0.005). This did not reach significance for UC, where only 6 UC patients had established MHD (MHD no median=0, MHD yes median=1, Mann-Whitney 146.5 p=0.267). Despite this, they were no more likely to remain in an active disease state at 12 months (overall X^2 2.36 p=0.125, UC X^2 2.03 p=0.16, CD X^2 0.27 p=0.60), progress to an AT within 12 months (overall X^2 0.008 p=0.930, UC X^2 0.59 p=0.44, CD X^2 1.7 p=0.19) or require inpatient care in the first 12 months (overall X^2 0.740 p=0.390, UC X^2 0.59 p-0.44, CD X^2 0.63 p-0.43).

Secondly, the relationship between baseline disk scores and one-year outcomes was explored. IBD patients with a higher overall disk score at baseline were more likely to have progressed to an AT (AT no; median baseline IBD disk score 45.5, AT yes; 63, Mann Whitney 1805 p<.001), have ongoing disease activity (Inactive disease 45.5, active 56, Mann-Whitney 1728 p<.001), have received inpatient treatment at some stage (no inpatient care; median 49, inpatient care; 60, Mann Whitney 1164 p=0.004) and have undergone a bowel resection (Resection no; median 52, resection yes; median 63, Mann Whitney 293 p=0.049) at the conclusion of their first year of treatment. Looking at UC patients in isolation, those that went on to require inpatient treatment presented with markedly increased disk scores compared to those who did not, as shown in ***Figure 3-18.*** The ability of the IBD disk score to separate the

patient groups on either side of these comparisons was compared to traditional disease activity indices for each subgroup, as shown in ***Table 3-10.*** In UC patients, the effectiveness of the IBD disk is marginally inferior when compared to the partial mayo score in predicting future advanced therapy (AT) use or need for inpatient treatment, but marginally superior in identifying those with persisting disease activity at 12 months. For CD, both the HBI and IBD disk score are less able to differentiate patient subgroups. The IBD disk is less able, when compared to the HBI, to identify CD patients who went on to require AT, have persisting disease activity at 12 months or require inpatient care. The prediction of AT utilisation in CD is of less value in the post PROFILE era (Noor et al., 2024) where any CD patients with moderate severe disease should progress to AT. Indeed, 49.5% of the included CD patients did within 12 months. However, identifying the need for these therapies in UC is less straight forward. Indeed, only 21% of UC patients progressed to AT within 12 months and therefore the ability of the IBD disk score to predict this is importance.

Table 3 - 10: Median (IQR), Mann-Whitney derived p values and AUC for all plotted values, comparing the ability of the baseline IBD disk score and traditional activity indices to differentiate future outcomes in CD and UC patients

	UC (n=71)		**CD (n=85)**	
	Partial Mayo	IBD disk	Harvey Bradshaw Index	IBD disk
Advanced therapy (AT) within 12m **UC AT 15/71 (21%)** **CD AT 43/85 (51%)**	No AT: 4 (2) AT: 7 (2) p<.001 AUC 0.832	No AT: 40 (32.25) AT: 57 (25) p=0.014 AUC 0.708	No AT: 6 (4) AT: 9 (4) p=<.001 AUC 0.744	No AT: 55 (24.5) AT: 64.5 (28) p=0.107 AUC 0.602
Active disease at 12m **UC Active 18/71 (25%)** **CD Active 35/85 (41%)**	Inactive: 4 (3) Active: 6 (3) p=0.032 AUC 0.672	Inactive 40.5 (34.25) Active: 53.5 (34.75) p=0.028 AUC 0.675	Inactive: 6 (4.25) Active: 9 (5) p=0.013 AUC 0.660	Inactive: 56 (32) Active: 62 (27) p=0.052 AUC 0.626
Inpatient (IP) care during first 12m **UC IP care 15/71 (21%)** **CD IP care 13/85 (15%)**	No IP care: 3.5 (2) IP care: 7 (1) p<.001 AUC 0.924	No IP care: 40 (32.25) IP care: 72 (31) p<.001 AUC 0.829	No IP care: 7 (4) IP care: 9 (4) p=0.149 AUC 0.626	No IP care: 56.5 (26.5) IP care: 55 (27) p=0.912 AUC 0.510
Resection within 12m **CD resection 6/85 (7%)**	NA (1 patient only)	NA (1 patient only)	No resection no: 8 (4) Resection: 7 (5.25) p=0.594 AUC 0.566	No resection: 56 (30) Resection: 68.5 (29.25) p=0.145 AUC 0.680

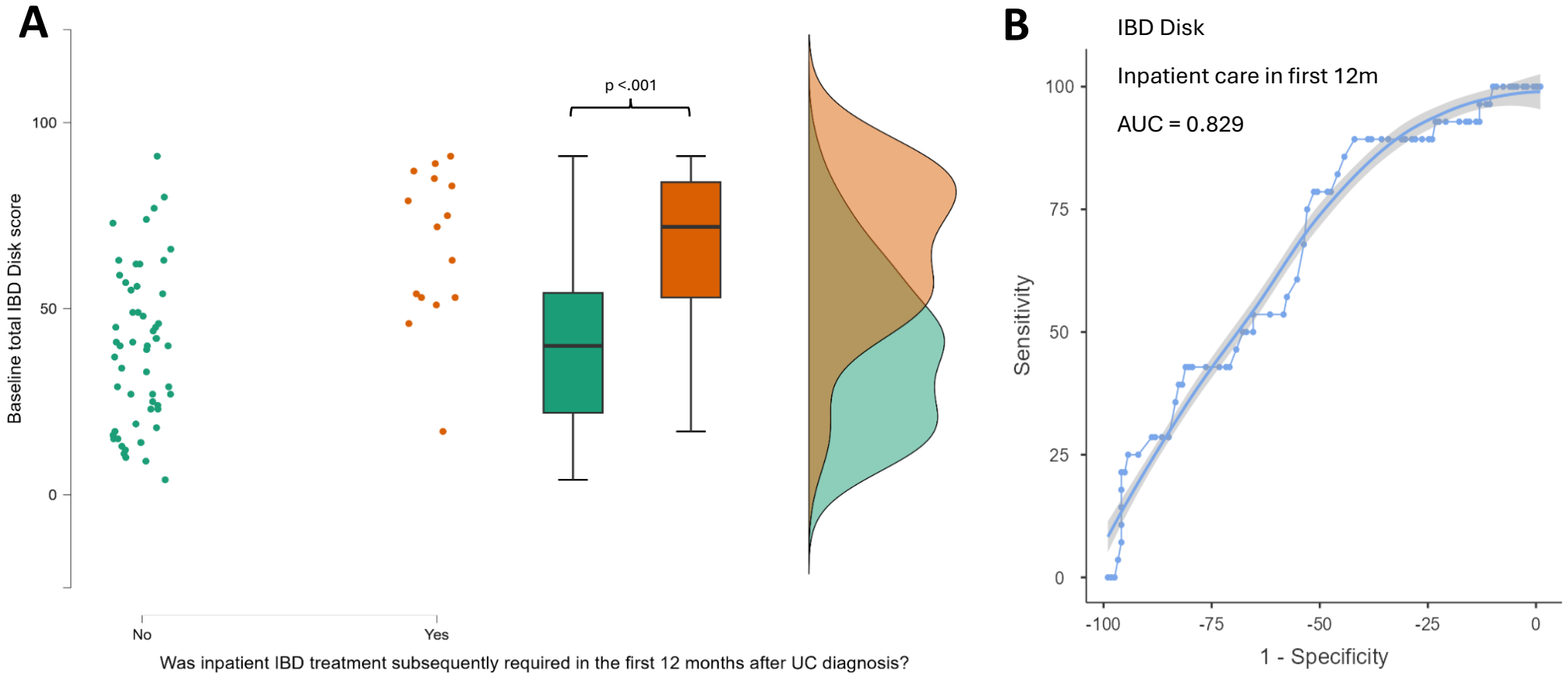


Figure 3 - 18: A raincloud plot demonstrating the significant elevation in total IBD Disk scores at baseline in those with UC that go on to be admitted to hospital for IBD treatment in the first 12 months after diagnosis, alongside a ROC curve for all IBD disk total scores.

A. This raincloud plot encompasses 71 UC patients of whom 15 required inpatient care for their colitis in their first 12 months after diagnosis. The median IBD Disk score at baseline was 40 (IQR 32.25) in those that did not required admission, and 72 (IQR 31) in those that did. This difference was highly significant on statistical testing (Mann-Whitney 144 p<.001)
B. The Area under the Curve for the ability of all IBD disk scores from UC patients to detect those that will need inpatient treatment in the first 12 months was 0.829. The optimal cut-point for this was a score of 51 (Sensitivity 86.67%, specificity 73.31%, Youden's index 0.599).

3.7.4 Discussion

The data presented signifies a novel evaluation of the IBD disk, both in terms of its implementation in a pre-diagnosis cohort of patients with IBD and the inclusion of patients in whom IBD is subsequently excluded. It is also acknowledged that obtaining disk scores when diagnostic uncertainty remained may have heightened factors such as anxiety, but this uncertainty was present across all included cohorts, and it allowed for the attainment of patient scores that were truly not influenced by knowledge of diagnosis at the first assessment (Massazza et al., 2022). In this context, we have been able to demonstrate the high prevalence of significant psychological symptoms across these cohorts, particularly in those who have IBD excluded and in CD relative to UC. Amongst those with IBD, IBD disk scores, particularly in the *'Emotions'* domain are strongly associated with HADS anxiety and depression scores. Indeed, an *'Emotions'* score of ≥7 identified all patients with HADS determined moderate or severe depressive symptoms.

The burden of symptoms, particularly in psychological domains, is striking in the non-IBD cohort. This group encompasses heterogenous diagnoses, but all were selected by at least transient elevation in FCP. Some of these transient illnesses were attributed to infection, but causative pathogens were rarely identified. Whilst further in-depth discussion is beyond the scope of this paper, it is a cohort that continues to increase in size and may warrant independent analysis after further follow up.

In UC, increased IBD disk scores at presentation correlated with biochemical and endoscopic disease activity. Furthermore, the disk was able to identify those patients

with UC at an increased risk of future adverse outcomes. This is particularly important given these adverse treatment outcomes are less frequent and often more challenging to predict in UC than CD. Outcomes predicted included an increased need for AT, inpatient treatment and persistently active disease after 12 months of treatment. This disk is comparable to the Partial Mayo score despite being self-administered and lacking the 'Physician's Global Assessment'. As such, in UC patients, disability and psychological disturbance appear to be key markers of disease severity and treatment outcome. Higher disk scores at presentation should prompt consideration of a more aggressive therapeutic approach with earlier treatment escalation.

Tracking IBD disk scores longitudinally has highlighted persisting disease activity as the key driver of enduring psychological disease burden, a finding we have demonstrated across both UC and CD. The differences driven by disease activity here are stark and further discounts diagnostic uncertainty as a key contributor to the differences observed in the presented analyses.

Patients with pre-existing MHDs appear to be exposed to different prescribing behaviours and are more likely to receive courses of oral corticosteroids, despite being no more likely to progress to AT or have ongoing disease activity after a year of treatment. Whilst this finding has been previously noted in active IBD, it remains noteworthy given the long-established propensity of steroids to exacerbate psychological symptoms in IBD patients (Fairbrass et al., 2022, Graff et al., 2009). Early identification of significant psychological symptoms, as the IBD disk can

facilitate, may help prevent this vicious cycle of steroid overuse being initiated in this group of patients who typically do not have an excess of other adverse treatment outcomes at 12 months post diagnosis. This data highlights that targeted psychological support and intervention may be able to positively impact patient care at IBD diagnosis, as well as previously demonstrated in those with active disease (Eccles et al., 2021, Regueiro et al., 2018).

The difference in size of the cohorts for each analysis, and the longitudinal cohort being less than half the size of the overall cohort is a key limitation. In IBD, those with more severe mental health diagnoses may be less likely to engage with clinical follow up (McQueenie et al., 2019) and this may skew the differences observed. In the non-IBD cohort, there is a lack of longitudinal data from the majority with repeated measures skewed towards those with a higher symptom burden more likely to require ongoing follow up after the exclusion of IBD. Finally, all scores are also derived from a single IBD centre which limits generalisability.

Utilisation of the IBD disk is not time consuming. Implementing it as a patient reported outcome measure from the outset in patients referred with suspected IBD could allow early identification of those who are likely to require more aggressive IBD treatment to obtain disease control. Alongside this, the disk can reliably detect clinically relevant depressive symptoms. It is a tool that can allow the more consistent delivery of the IBD standards regarding the holistic assessment of psychological disease burden.

3.8 Conclusions

In an adverse climate within the NHS, our Inception clinic has markedly reduced secondary care delays associated with IBD diagnosis. The implementation of the pathway associated with this project has transformed early IBD care within one of the largest hospital trusts in the UK, with roll out now across all sites.

Despite our efforts and multiple national campaigns, delays remain regarding diagnosis. Better utilisation of symptoms and FCP at triage can ensure resources are given to those most at risk of IBD. As we plan new care pathways, our data would support a move away from traditional approaches. A second FCP ≥220ug/g, regardless of the first result, was a more accurate predictor. Coupled with key clinical symptoms, the formulation of reliable self-referral pathways based on patient-initiated home FCP testing could allow even more rapid diagnosis and treatment initiation. As a result of the work here I am supporting the implementation of a pilot self-referral pathway in Devon which, if successful, we will attempt to validate in Birmingham.

We have validated that IBD disk as a screening tool for significant psychological symptoms in clinic but also highlighted value of the disk and disease associated disability as a predictor of early disease course, particularly in UC. Once IBD diagnosis is established, active disease is the key driver in those with a persisting high psychological symptom burden.

CHAPTER 4: The role of β-galactoside binding lectins at IBD onset

4.1 Abstract

Introduction

β -galactoside binding lectins, or 'galectins' are a family of glycan binding proteins with key roles in the mediation of inflammatory pathways and functions in the regulation of host-microbial interactions. Whilst numerous galectins have been evaluated in IBD, this has seldom been undertaken at IBD inception and never longitudinally. An unpublished local pilot study demonstrated elevated levels of GAL-3 and -9 in IBD patients relative to HC.

Methods

Patients referred with suspected IBD were recruited prior to the initiation of treatment. Serum was taken for ELISAs focused on GAL-1, -3 and -9. Clinical indices and outcomes were collected prospectively. Where possible, post treatment longitudinal samples were collected.

Results

Baseline galectin levels were measured at IBD onset (n=128, CD=69, UC=59) and in controls (SC=21, HC=38). 57 IBD patients provided post treatment follow up samples, with 10 providing samples at three time points.

Significant elevations were observed across UC and CD in GAL-3 (Dunn test, $p_{holm}<0.001$ for CD and UC) and GAL-9 (CD $p_{holm}<.001$, UC $p_{holm}=0.003$) relative to HC. No differences were seen between IBD and SC. GAL-9 correlated with indices including CRP ($r_s=0.373$ $p<0.001$) and WCC ($r_s=0.324$ $p<0.001$), whilst GAL-1 correlated with FCP ($r_s= -0.232$ p 0.036) and SESCD ($r_s=-0.249$ p 0.045). Baseline

GAL-9 levels were higher in those progressing to AT (p=0.002) and in those with treatment non-response at 6 months (p<.001). In a regression model, GAL-9 levels significantly outperformed traditional biochemical indices (Hb, CRP, WCC, FCP, platelets, ferritin) at predicting treatment non-response at 6 months (n=97, LR 10.22 p=0.001).

Treatment initiation resulted in significant reductions in both GAL-3 (Wilcoxon p=0.007) and GAL-9 (Wilcoxon p=0.003). In a linear model, GAL-9 levels were shown to be significantly influenced by systemic corticosteroid use regardless of disease state (t=2.03 p 0.048).

Conclusions

Significant elevations in GAL-3 and GAL-9 are seen in IBD relative to HC, but they cannot differentiate IBD from SC. GAL-9 strongly associates with inflammatory indices and is shown to accurately reflect disease state longitudinally. Baseline GAL-9 levels are elevated in those going on to require AT and are predictive of early treatment outcomes after the initiation of IBD treatment. If validated in multi-centre studies, measurement of GAL-9 could become a valuable biomarker for disease stratification at IBD onset.

4.2 Introduction

The literature review presented in the introduction highlighted some strong signals warranting further study in IBD, particularly in a pre-treatment cohort. Our decision-making regarding choice of galectin was made far easier because of unpublished pilot work that took place at the University of Birmingham between 2016 – 2018. I personally did not contribute to this work but have been given permission to utilise the data by Dr. A. Iqbal and Dr S. Tull. Furthermore, limited analyses had been undertaken because of a paucity of metadata, comprising only diagnosis. I have been able to revisit this patient cohort, retrospectively collect metadata and attempt additional analyses to add some depth to the conclusions previously made. Ethical approval for this work was afforded by an existing protocol allowing the profiling of patients with established gastrointestinal diseases as earlier described in the methods (Yorkshire & The Humber – Bradford Leeds Research Ethics Committee [REC reference: 16/YH/0100]).

4.3 Results from the galectin pilot study

This work was undertaken in line with the earlier methodology, utilising the same ELISA kits and techniques. Analyses were focussed upon GAL-1, GAL-3 and GAL-9. The patient cohort was a heterogenous group of IBD patients which varying disease activity and treatment histories, skewed towards more active or extensive disease. Comparisons were made with an unmatched group of HC. The cohorts are summarised in ***Table 3-1***. All galectin values expressed through this section are ng/ml.

Table 4 - 1: Patient demographics within the galectin pilot study

	Crohn's Disease **(n = 18*)**	Ulcerative Colitis **(n = 34*)**	Healthy controls **(n = 22)**
Age, median(IQR), y	32.5 (14.3)	39 (20)	27.5 (6.75)
Sex, n (%) male	8 (50)	15 (52)	64
Duration of diagnosis, median(IQR), y	8 (7.75)	6 (10)	n/a
Disease location*, n. (%)	Ileal: 1 (6) Colonic: 2 (13) Ileocolonic: 13 (81)	Proctitis: 3 (10) Left sided: 8 (28) Extensive: 18 (62)	n/a
Disease behaviour, n. (%)	Non-stricturing, non-penetrating: 9 (56) Stricturing: 2 (13) Penetrating: 5 (31)	n/a	n/a
Disease state, n. (%)			
Active	9 (56)	19 (66)	n/a
Remission	7 (44)	10 (34)	
Treatment, n. (%)			
Mesalazine	2 (13)	27 (93)	n/a
Steroids	3 (19)	8 (28)	
Immunomodulator	8 (50)	7 (24)	
Advanced Therapies	5 (31)	7 (24)	
Previous surgery, n (%) yes	6 (37.5)	0	n/a
Laboratory parameters, median(IQR)			
Hb, g/l	135 (25)	137 (14)	n/a
Platelets, 10*9/L	278 (129)	255 (126)	
White cell count, 10*9/L	7.45 (3.03)	6.7 (2)	
CRP, mg/L	4.5 (13)	4 (3.5)	
Faecal Calprotectin, ug/g**	535 (1194)	585 (475)	

*Full metadata could not be retrieved for 5 UC and 2 CD patients ** Faecal calprotectin was only available for 8 UC and 6 CD patients

4.3.1 Differences between patient groups

Within the cohort, strong correlations were seen between the levels of all galectins measured ***(Figure 4-1)*.** When looking purely at demographics and considering important factors to correct for, all galectins measured were seen to positively correlate with patient age (GAL-1 r_s=0.315 p=0.016, GAL-3 r_s=0.308 p=0.016, GAL-9 r_s=0.541 p<0.001). This is noteworthy given the increased age associated with the UC cohort. No significant differences were seen with patient sex. In IBD, no correlations were seen with disease duration.

Comparing grouped IBD and HC, significant differences were seen between GAL-3 (median 7.61 vs 5.55, Mann-Whitney U 307 p 0.002) and GAL-9 (median 5.89 vs 4.08, Mann-Whitney U 254 p<0.001), but not GAL-1 (27.53 vs 24.55 Mann-Whitney U 441 p 0.244). Amongst disease subgroups, the differences remained significant between both UC and CD when compared with HC, but not when compared against each other. The median GAL-1 level was non-significantly increased in CD patients (29.33 vs 24.55 healthy and 25.88 UC p_{holm}=0.098). Plots of this data are presented in ***Figure 4-2***.

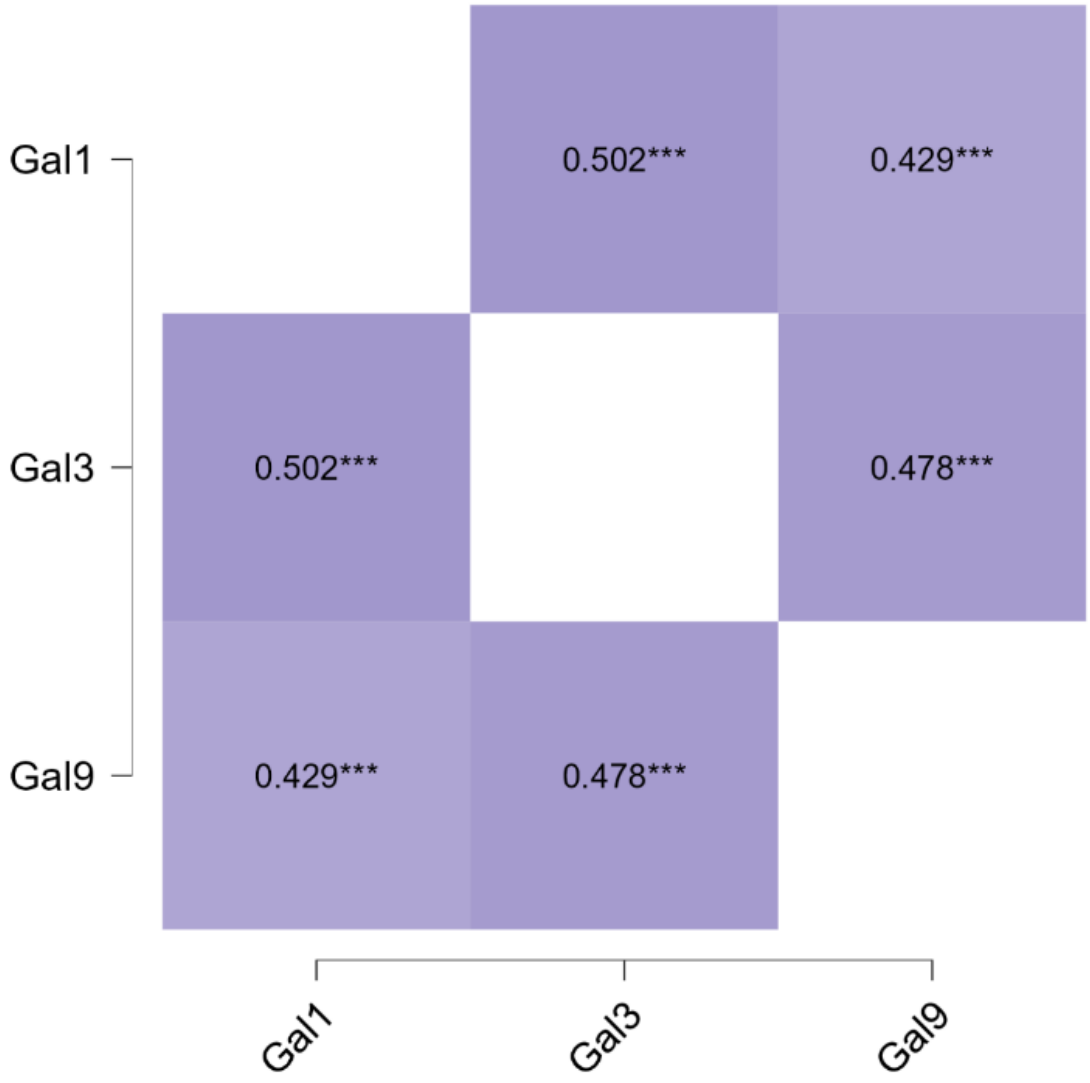
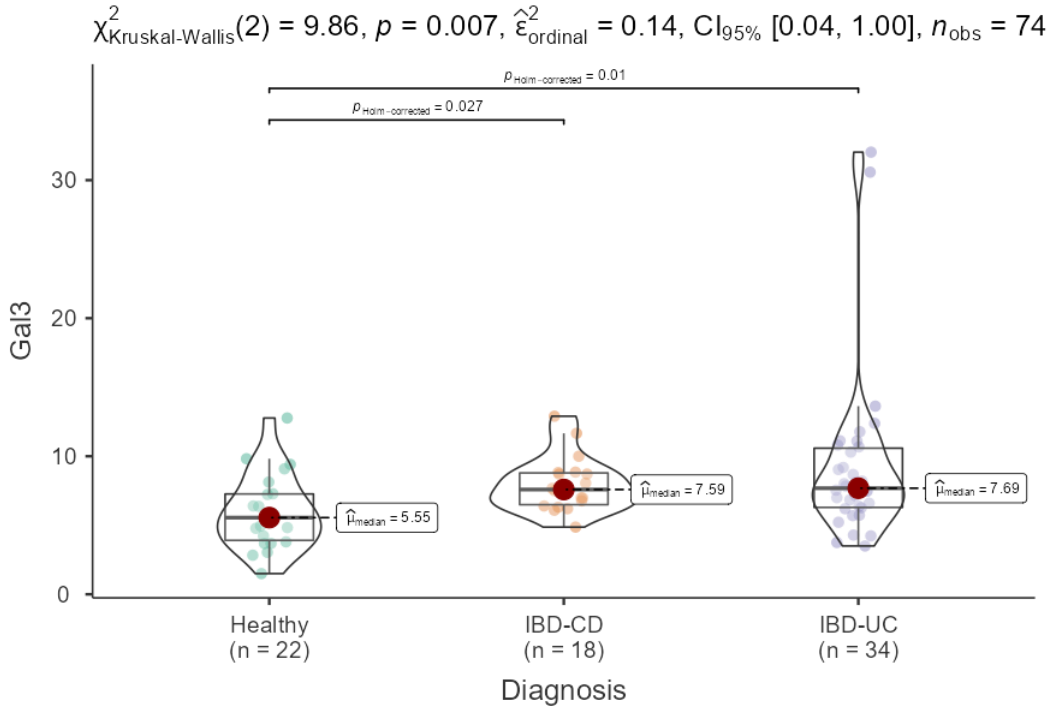


Figure 4 - 1: The strong relationship between GAL-1, -3 and -9 levels

When including the entire cohort (n=74), strong correlations were seen between all of the three galectins measured (Spearman's Rho, GAL-1 to GAL-3 r_s= 0.50 p<0.001, GAL-1 to GAL-9 r_s= 0.43 p<0.001, GAL-3 to GAL-9 r_s= 0.48 p<0.001)



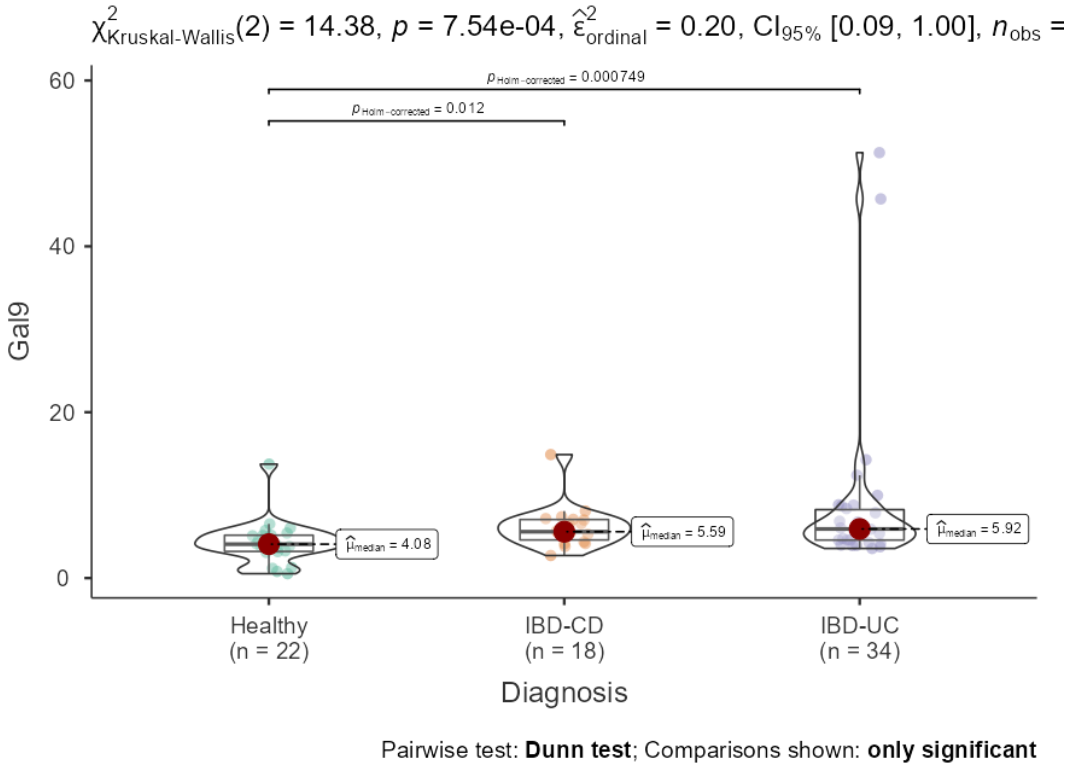


Figure 4 - 2: GAL-3 and GAL-9 levels split by patient group
As determined using Kruskal-Wallis and the Dunn test as a pairwise measure, significant differences were seen with healthy controls (n=22) for both GAL-3 and GAL-9. Levels of both of there was marginally higher in UC (n=34, Kruskal-Wallis[2], Dunn, GAL-3 p=0.01, GAL-9 p=0.0007) and CD (n=18, Kruskal-Wallis[2], Dunn, GAL-3 p=0.027, GAL-9 p=0.012). There were no significant differences between CD and UC.

4.3.2 Differential galectin levels with disease activity and severity

Amongst IBD, significant differences were seen in GAL-3 levels between those with active disease and those in remission (Active median 8.44 vs remission 6.98, Mann-Whitney U 150 p=0.041). However, when this difference is examined more closely using Kruskal-Wallis(2), it is almost entirely attributable to differences seen in those UC (p=0.026) and not CD (p=0.916). No significant differences were seen with GAL-1 or GAL-9.

When comparing galectin levels to other laboratory indices, there were only weak signals to suggest association. GAL-1 was seen to correlate negatively with haemoglobin level (Spearman's Rho r_s=0.359, p=0.017), whilst GAL-3 was seen to correlate with Platelet count (r_s=0.350, p=0.018). Associations between GAL-3 and haemoglobin (r_s=-0.289 p=0.054) or white cell count (r_s=0.263 p=0.081) did not reach statistical significance. There were no associations with faecal calprotectin, though that was an underutilised tool at the time that this pilot work was completed. It was not possible to accurately collect disease activity scores from retrospective case note review as documentation was not sufficient.

Median GAL-3 levels were non-significantly elevated in stricturing (9.03) and penetrating (8.06) Crohn's disease compared to uncomplicated disease (6.98). In UC, there were no differences in GAL-1, -3 or -9 levels according to the Montreal severity grading attributed to each patient. From the CD cohort, 6 patients had

previously undergone a surgical resection. These patients tended to lower GAL-9 levels, though this was not significant (Mann-Whitney U 172.5 p=0.066).

4.3.3 Differential galectin levels with treatment modality

Given most patients with CD were not on Mesalazine and the majority with UC were, comparing galectin levels according to utilisation of this medication are not meaningful. There were no significant differences across all types of galectins according to Immunomodulator (azathioprine, mercaptopurine or methotrexate) use. However, when grouping IBD subtypes together, there was a significant reduction in GAL-3 level in patients who were on biologic treatment vs those who were not (median 8.2 vs 6.99, Mann-Whitney U 105 p=0.017). Differences were seen in both disease subgroups, but when evaluated in isolation, this only reached significance in the UC cohort (p=0.030). This difference cannot be explained by disease activity alone, with a greater proportion of those on AT deemed to have active disease (75%) than those who were not (58%). In a binomial regression model (McFadden's pseudoR^2 = 0.260 p=.001) with GAL-3 as a covariate and disease state as a factor, GAL-3 level alone was able to predict if the patient was on a biologic with 76% accuracy (AUC 0.819). The use of corticosteroids at the time of blood sampling did not result in significant differences across any of the galectins measured.

4.3.4 Early signals and limitations from the pilot cohort

There are some clear signals from this cohort, though the data has the same limitation of many of the other studies in the area with a heterogenous patient group in terms of treatment effects. However, in depth analysis has highlighted additional

reductions in GAL-3 in association with biologic therapy that have not previously been seen in an IBD cohort. This change was present despite a higher prevalence of patients with active disease in this cohort, something associated with increased GAL-3 levels when analysing the cohort as a whole. Outside of this, GAL-3 increases in IBD relative to HC, as previous studies have shown. This is the first study to demonstrate increased GAL-9 levels in the serum of patients with IBD relative to HC.

4.4 Results from the Inception cohort

All IBD patients included from this point forwards were naïve to treatment at the time that their baseline serum was taken for the galectin ELISAs. There is a comparative cohort of HC in addition to an SC cohort of patients seen via the IBD inception pathway in whom an IBD diagnosis was excluded.

4.4.1 The study cohort

Participant characteristics are summarised in ***Table 4-2***. Complete metadata was available for the majority, with small numbers missing from HC and patients recruited at BHH. The SC cohort included 19 patients deemed to have functional gastrointestinal symptoms (e.g. IBS) and 2 with haemorrhoidal bleeding. As discussed in the methods, GAL-1, -3 and -9 were the focus here. However, a subgroup also had GAL-10 and TIM-3 measured. Latterly, we have undertaken cytokine analyses which are presented here only in relation to galectin expression.

Table 4 - 2: Patient demographics and disease indices for the IBD Inception galectin Cohort.

	CD **(n = 69)**	UC **(n = 59)**	Symptomatic control **(n = 21)**	Healthy control **(n = 39)**
Age, median (IQR), y	30 (17.3)	35.5 (19.8)	34 (15)	26 (7)
Sex, n. (%) male	25 (42)	29 (58)	10 (48)	17(52)
Ethnicity, n. (%)				
Asian	18 (26)	8 (14)	1 (5)	4 (13.3)
Black	6 (9)	5 (8)	1 (5)	1 (3.3)
White	45 (65)	46 (78)	19 (90)	25 (83.3)
BMI, median (IQR)	24.5 (8.06)	24.3 (4.6)	29.6 (10.2)	25 (5.2)
Smoking, n. (%) current smoker	11 (17)	4 (7)	4 (14)	Not known
Disease location, n. (%)	Ileal: 31 (45) Colonic: 16 (23) Ileocolonic: 22 (32)	Proctitis: 13(28) Left sided: 12 (25) Extensive: 22 (47)	n/a	n/a
Disease Behaviour / Severity, n. (%)	Non-stricturing, non-penetrating: 53 (79) Stricturing: 10 (15) Penetrating: 4 (6)	Mild: 20 (43) Moderate: 21 (45) Severe: 6 (13)	n/a	n/a
Faecal Calprotectin, median (IQR), ug/g				n/a
At referral	676 (1210)	1782 (1666)	452 (415)	
At time of serum sampling	516 (914)	743 (1992)	21 (124)	
Biochemistry, median (IQR)				n/a
Haemoglobin, g/L	133 (25.5)	135 (22.8)	146 (18)	
Platelets, 10*9/L	345 (190)	327 (145)	279 (113)	
White cell count, 10*9/L	7.9 (3.51)	6.95 (2.6)	6.3 (5.75)	
CRP, mg/L	7.5 (13)	2 (5.75)	3 (3)	
Clinical severity, median (IQR)	Harvey Bradshaw Index: 8 (5)	Partial Mayo: 4 (3)	n/a	n/a
Endoscopic severity, median (IQR)	SESCD: 6 (8.5)	UCEIS: 4 (4)	n/a	n/a

4.4.2 Does the strength of inter-galectin relationship remain at disease onset?

At disease onset, significant correlations are observed between GAL-9 and both GAL-1 and -3 (GAL-1 to GAL-9 r_s=0.28 P<0.001, GAL-3 to GAL 9 r_s=0.31 p<0.001). However, no significant association remained between GAL-1 and -3 (r_s =0.117 p 0.112). Significant associations between age and galectin level were only maintained for GAL-1, but to a lesser extent (r_s=0.264 p<0.001, ***Figure 4-3***).

4.4.3 Differences in galectin level at IBD onset

At baseline, no significant differences were seen in GAL-1 between patient groups (Kruskal-Wallis (3) 1.26 p 0.740, no significant pairwise correlation). Marginally higher levels were seen in the control cohorts (HC – median 24.22; SC – 25.08) than the IBD cohorts (CD – 23.05; UC 23.46). For GAL-3 significant differences were seen between both IBD cohorts and HC. However, GAL-3 levels were also significantly higher in SC than HC (***Figure 4-4***).

The same findings were replicated in GAL-9 measurements. Again, it was possible to demonstrate significant differences between both IBD subgroups and HC, but SC also had significantly elevated GAL-9 level compared to HC. No significant differences were seen between IBD and SC regarding GAL-9, though levels were substantially higher in patients with CD than UC (Dunn test, p<0.001, ***Figure 4-5)***.

TIM-3 and GAL-10 were included in the first set of ELISAs. For both, no significant differences between IBD or controls were observed and further analysis not pursued.

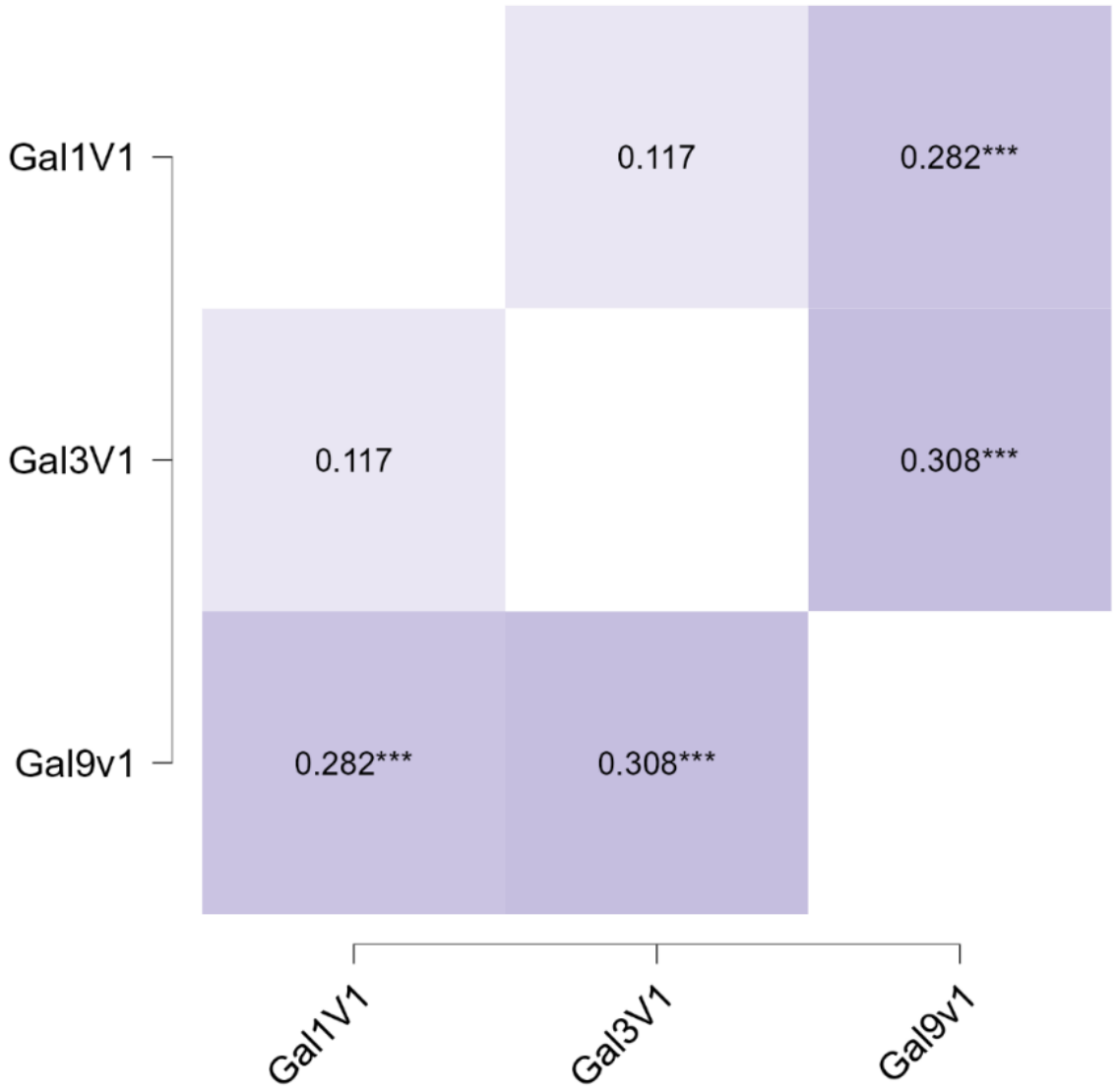


Figure 4 - 3: Correlation Matrix of galectin levels in IBD Subgroups.

Across the whole cohort (n=187), significant correlations remained between GAL-1 and GAL-9 (r_s=0.28 p<0.001) and GAL-3 and GAL-9 (r_s=0.31 p<0.001) when assessed using Spearman's Rho. When comparing subtypes of IBD, slightly differential behaviours were seen. Patients with Crohn's disease (n=69) showed stronger correlations between GAL-3 and GAL-9 (r_s=0.37 p_{holm}<0.001), whilst Ulcerative colitis (n=59) had a stronger correlation between GAL-1 and GAL-9 (r_s=0.41 p_{holm}<0.001).

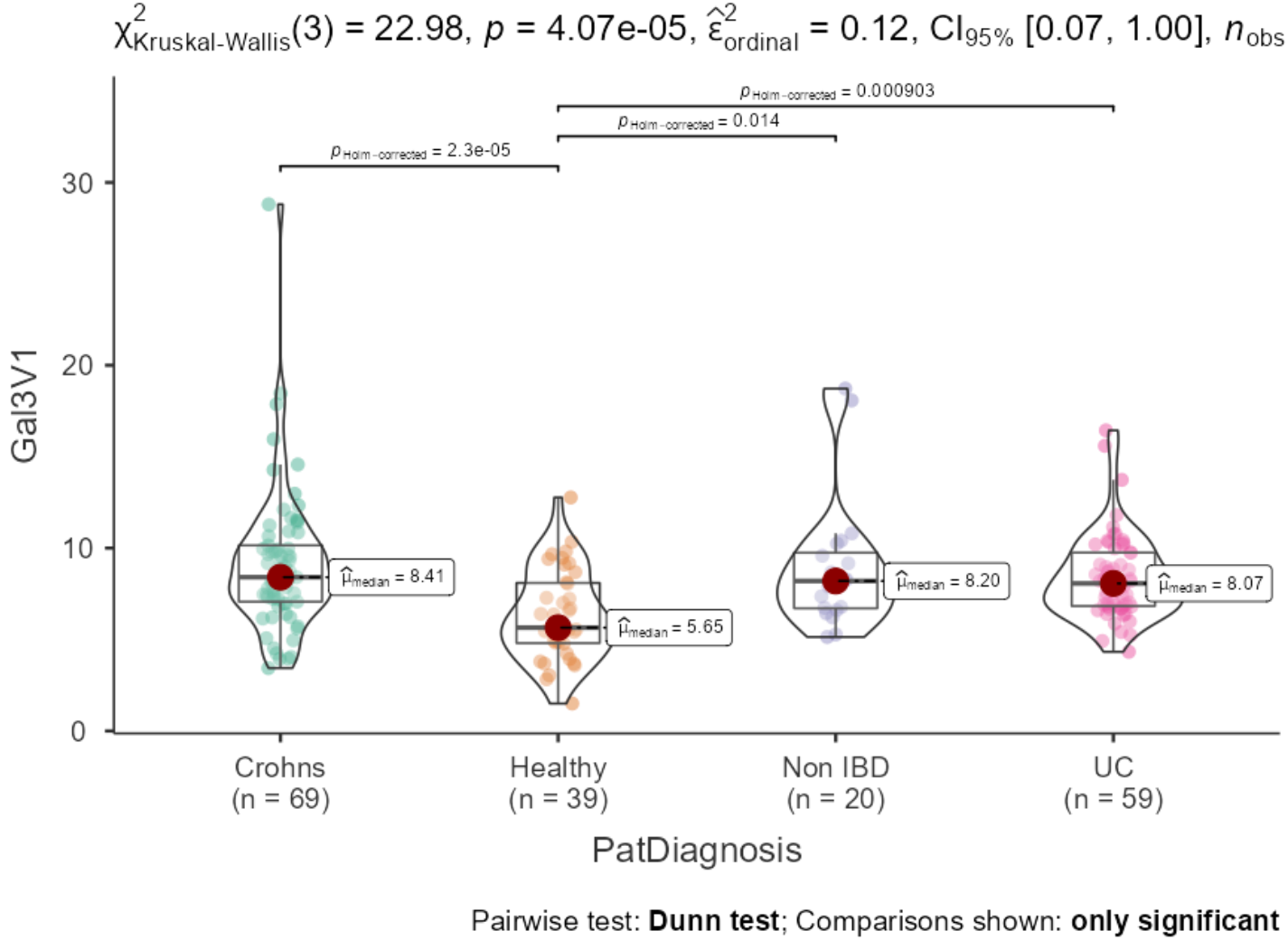


Figure 4 - 4: Differences in GAL-3 level at IBD onset

The highest overall GAL-3 levels were found in patients with CD (n=69) whilst the lowest levels were in healthy individuals (n=39). Using Kruskal-Wallis(3) and a pairwise Dunn test, significant differences was seen between healthy individuals and all patient groups, including symptomatic individuals without IBD (n=20). In CD, the difference was highly significant (p_{holm}= 0.00002), whilst UC (n=59, p_{holm}=0.0009) and Non IBD (p_{holm}=0.014) were also relevant. No significant differences were seen between IBD types or between IBD patients and symptomatic individuals without IBD.

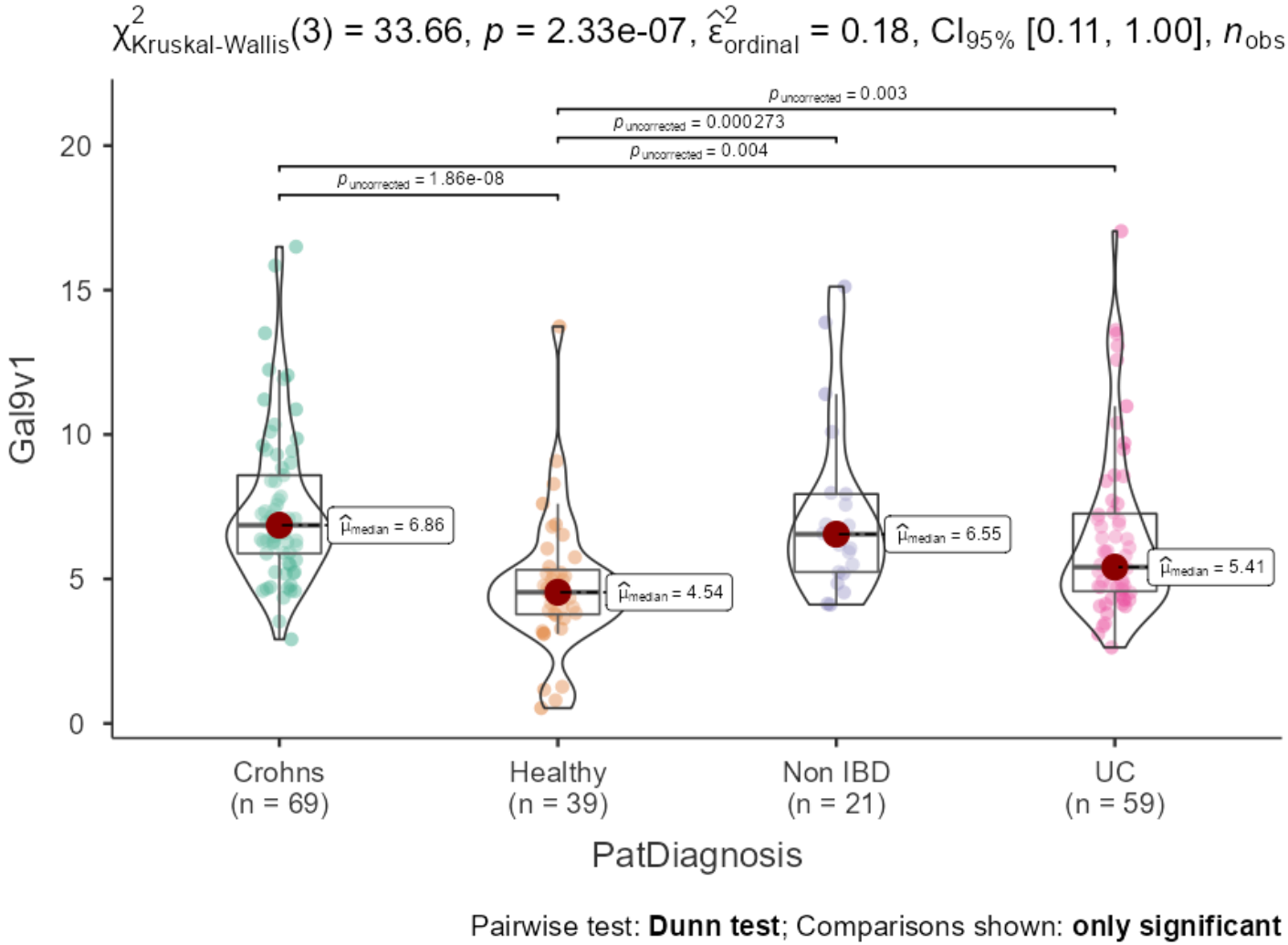


Figure 4 - 5: GAL-9 levels at IBD onset and amongst control cohorts

GAL-9 levels were also analysed using Kruskal-Wallis(3) and Dunn test for pairwise comparisons. The cohort included results for 69 CD, 59 UC, 39 Healthy and 21 Non IBD patients. Patients with CD had the highest GAL-9 levels across all the cohorts. The levels in CD were significantly higher in patients with UC (Dunn p=0.004) and healthy controls (Dunn p=0.00000001). Levels in both UC (Dunn p=0.003) and symptomatic controls (Dunn p=0.0003) were higher than in healthy individuals.

4.4.4 Associations with measures of disease activity and inflammation

There were several statistically significant correlations between galectins and other inflammatory indices, some of which have not previously been documented in IBD. GAL-1 was shown to have a weak negative association with FCP when measured on the same day as serum sampling (r_s= -0.232 p=0.036) and a weak positive association with Haemoglobin (r_s=0.198 p=0.017). GAL-3 levels weakly correlated with white cell count (r_s=0.165 p 0.048) and calcium level (r_s=0.244 p=0.009). The association with platelet count seen in the pilot data was not present. GAL-9 was associated with several different biochemical indices of inflammation and proinflammatory cytokines, as shown in ***Figure 4-6.***

GAL-1 continued to demonstrate a closer association with mucosal disease activity in CD, with levels showing a weak negative association with the SESCD score (r_s=0.249 p 0.045) and HBI (r_s=-0.251 p 0.039). Associations with disease activity indices were not replicated with GAL-1 in patients with UC, whilst GAL-3 and GAL-9 showed no significant relationships with activity indices in UC or CD.

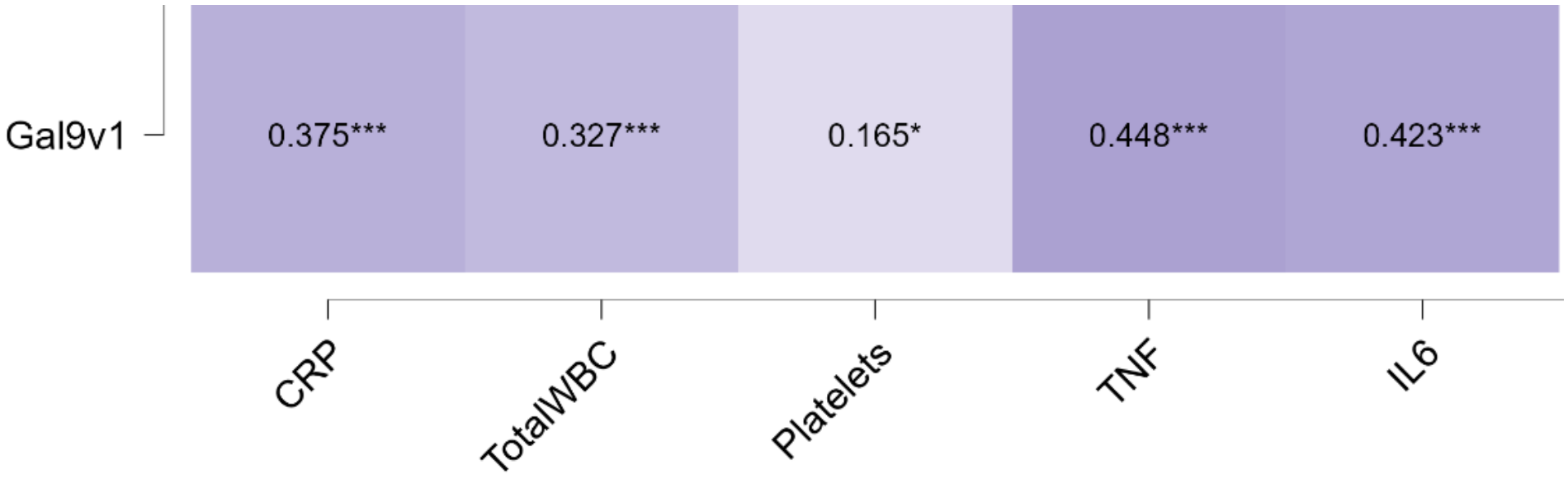


Figure 4 - 6: Heatmap of correlations between GAL-9, typical clinical biochemical indices and proinflammatory cytokines

Using Spearman's Rho, statistically significant associations were seen between GAL-9 and several indices associated with systemic inflammation, including white cell count (n=147 r_s=0.327 p=<0.001), CRP (n=148, r_s=0.375 p=<0.001) and to a lesser extent platelet count (n=147, r_s=0.177 p=0.032). Strong associations were also observed between GAL-9 and both TNF (n=74 r_s=0.448 p=<0.001) and IL-6 (n=68, r_s=0.423 p=<0.001). Outside of associations likely owing to inflammatory pathways, GAL-9 also significantly correlated with alkaline phosphatase (r_s=0.382 p<0.001).

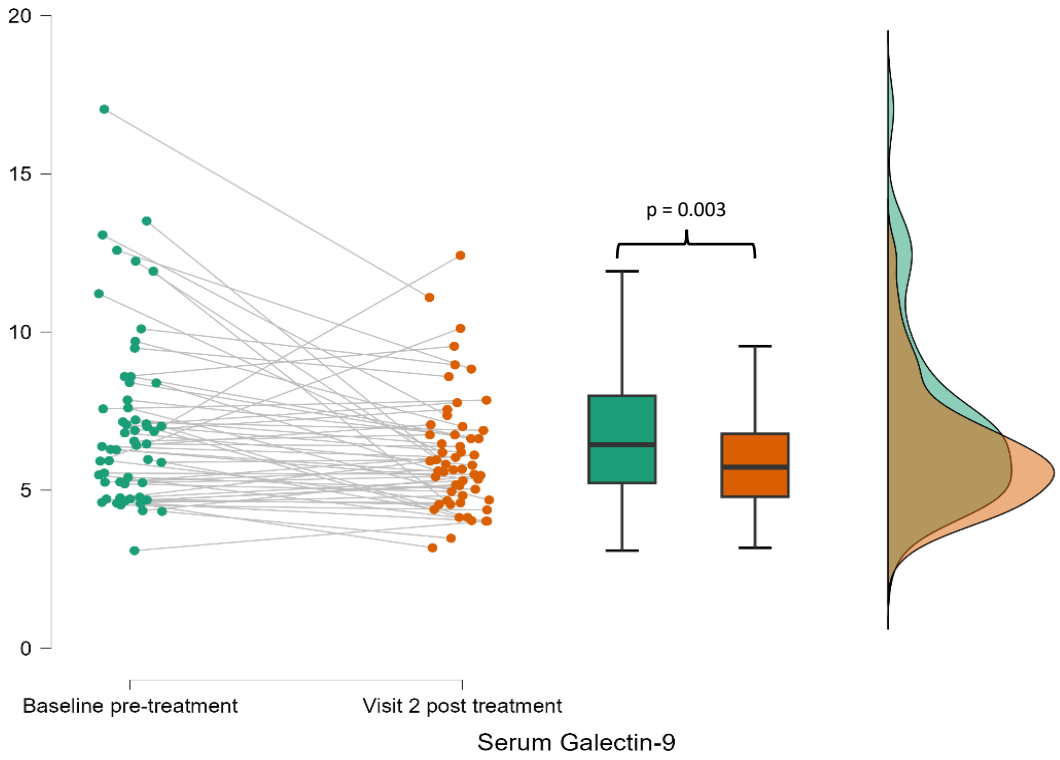
4.4.5 How does treatment initiation impact galectin expression?

31 patients with CD and 26 with UC provided a repeat galectin level during follow up, post treatment visit (visit 2). Of these, 6 CD and 4 UC patients, typically those with more difficult disease requiring further outpatient review, provided a third sample (visit 3). Looking at the post treatment samples together, there were no significant differences in the levels seen between CD and UC across any of the three galectins measured. However, treatment initiation manifested several significant differences when looking at paired samples. Across IBD, statistically significant reductions were seen pre and post treatment in GAL-3 and GAL-9 (***Figure 4-7***). No changes were seen in GAL-1 (Median 22.94 vs 22.82, Wilcoxon 864 p=0.593).

In IBD subgroups, the changes in GAL-3 and GAL-9 showed similar directions, though to different extents. The fall in GAL-9 was statistically significant in UC (Baseline median 6.44 vs 5.86 , Wilcoxon rank 271 p=0.014) but was not quite in CD (Baseline median 6.42 vs 5.61, Wilcoxon rank 325 p=0.058). This relatively small difference occurs in the context of a higher proportion of patients continuing to have active disease at visit 2 in the CD cohort (69%) compared to UC (41%). Indeed, those with CD reaching an inactive disease state did see a marginally greater fall in GAL-9 (to a median of 5.42). With GAL-3, despite the increased proportion of patients with active disease, a more pronounced change was seen in CD (Baseline median 8.05 vs 6.91, Wilcoxon 312 p=0.013) than UC (Baseline median 7.49 vs 7.37, Wilcoxon 223 p=0.24). In CD, the median GAL-3 level fell by 0.87 at visit 2 in those with persisting active disease, whilst for those reaching an inactive state it fell by 1.09. Despite this, in the 9 patients with persisting active UC, the median GAL-3 level increased by 0.64 (Baseline median 7.31 vs 7.95) as compared to a fall of 0.73

(Baseline median 7.56 vs 7.24) in those reaching an inactive state. In the visit 2 cohorts, overall differences between GAL-3 (Mann-Whitney U 282 p 0.8) or GAL-9 (Mann-Whitney U 235 0.18) according to disease activity do not reach statistical significance. This differences according to disease state are shown in ***Figure 4-8.***

At first glance, the differences here could be taken to reflect a dissociation of GAL-9 levels from systemic inflammatory markers after the initiation of treatment. Whilst significant at diagnosis, GAL-9 levels show no correlation with CRP (Spearman's Rho r_s=0.028 p 0.85) or WCC (r_s=0.051 p 0.73) post treatment. For GAL-3 however, the relationship seen with WCC prior to treatment strengthens (r_s=0.344 p=0.017) whilst a new weak relationship with FCP does not quite reach significance (r_s= -0.325 p=0.061). Though initially surprising, these differences appear to be explained primarily by corticosteroid use. A mainstay of early IBD therapy to induce remission, 31% of patients are on either Budesonide or Prednisolone at the Visit 2 sample timepoint. To demonstrate the impact of this, a multiple linear regression model was developed for the impact of disease state and corticosteroid use on GAL-9. This is presented in ***Figure 4-9.*** The overall model was significant (R2 0.149, F(2,46) 4.04 p 0.024). When viewed in this way, active disease still significantly associated with increased GAL-9 levels (t 2.34, p 0.024) and steroid use associated with significant falls in GAL-9 (t = -2.03 p 0.048). These associations are not seen with GAL-1 or -3.



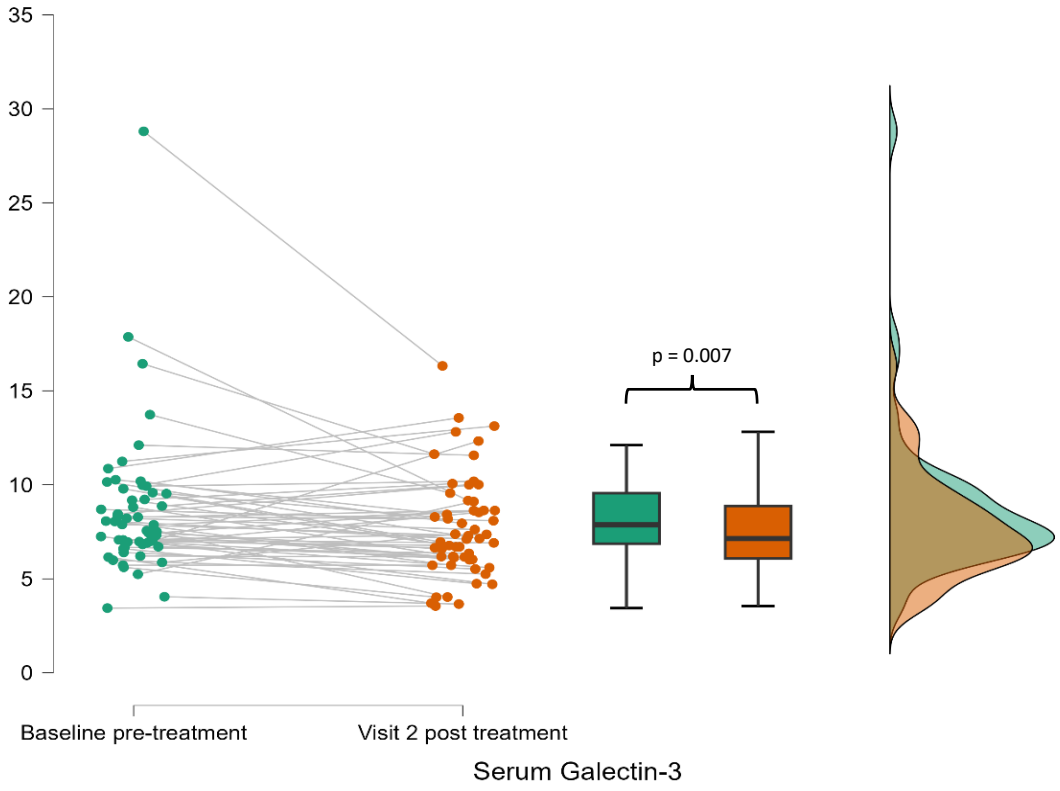


Figure 4 - 7: Impacts of treatment initiation on GAL-3 and GAL-9

Across all IBD patients with baseline and post treatment samples (GAL-3 n=55, GAL-9 n=56), statistically significantly different reductions are seen after the initiation of treatment in both GAL-3 (Median 7.87 vs 7.14, Wilcoxon rank 1054 p=0.007) and GAL-9 (Median 6.44 vs 5.73, Wilcoxon rank 1169 p=0.003).

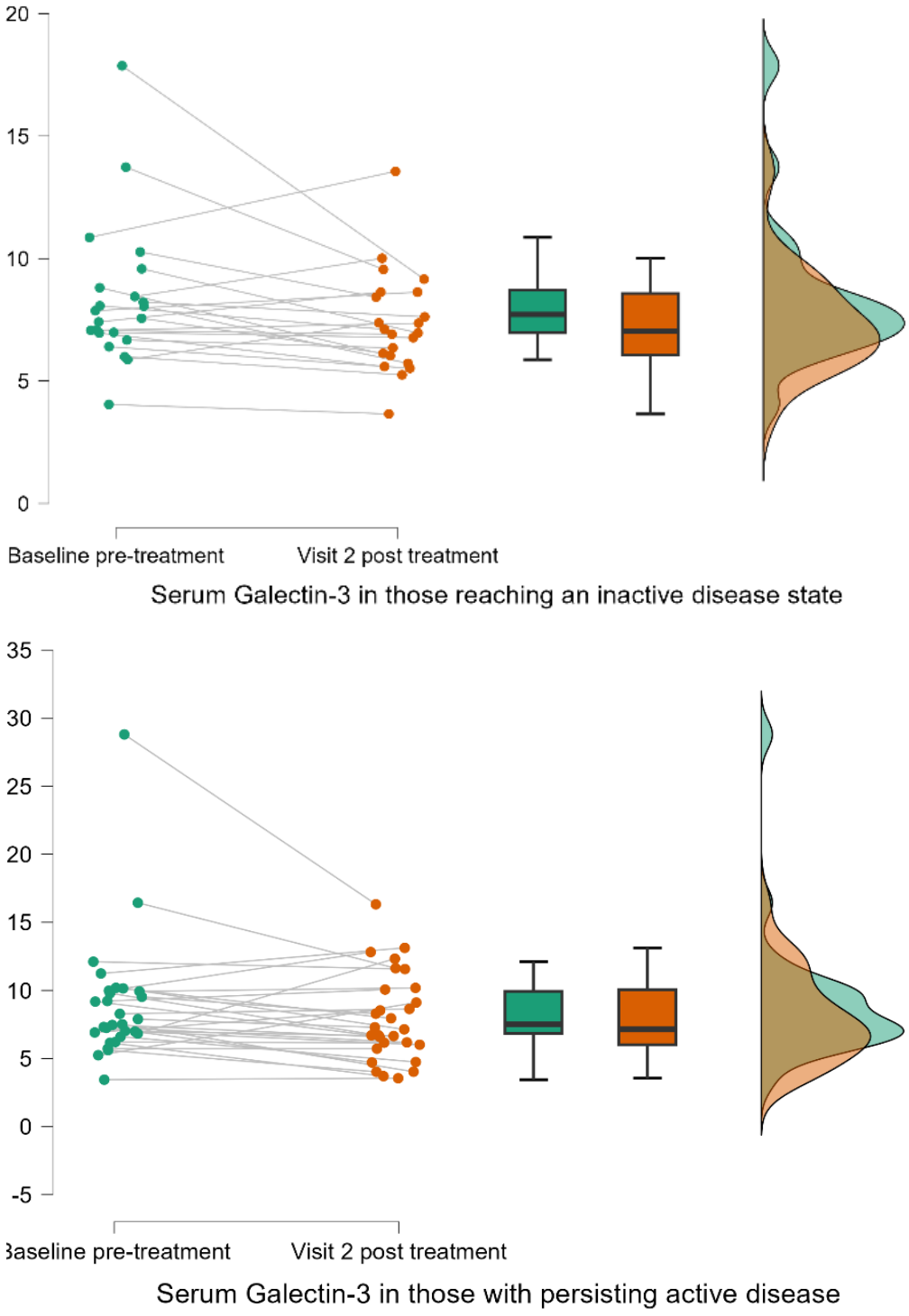


Figure 4 - 8: Repeated measures plots of GAL-3 levels, stratifying patients by disease state (active vs inactive) at the time of obtaining the visit 2 post treatment blood sample.

Median, IQR and spread of repeat measures is shown for IBD patients (n=47; CD=26, UC=21. 7 missing activity assessment excluded). The plots split those who remained in an active disease state (n=27, median 7.51 baseline vs 7.14 repeat) and those who reached an inactive disease state (n= 20, median 7.71 vs 7.04). In UC, the 9 patients with persistent active disease demonstrated an increase in GAL-3 by 0.64 (Baseline median 7.31 vs 7.95) as compared to a fall of 0.73 (Baseline median 7.56 vs 7.24) in those reaching an inactive state. In this underpowered cohort, none of these early signals achieved statistical significance. Whilst acknowledging that this is a parametric test, a repeated measures ANOVA with a post hoc comparison of active and inactive disease did not show significant differences in GAL-3 level (Mean difference 0.57, p_{holm}= 0.52). Furthermore, when considering the visit 2 samples in isolation, disease state did not result in a significant difference in GAL-3 level (Mann-Whitney U, p=0.8).

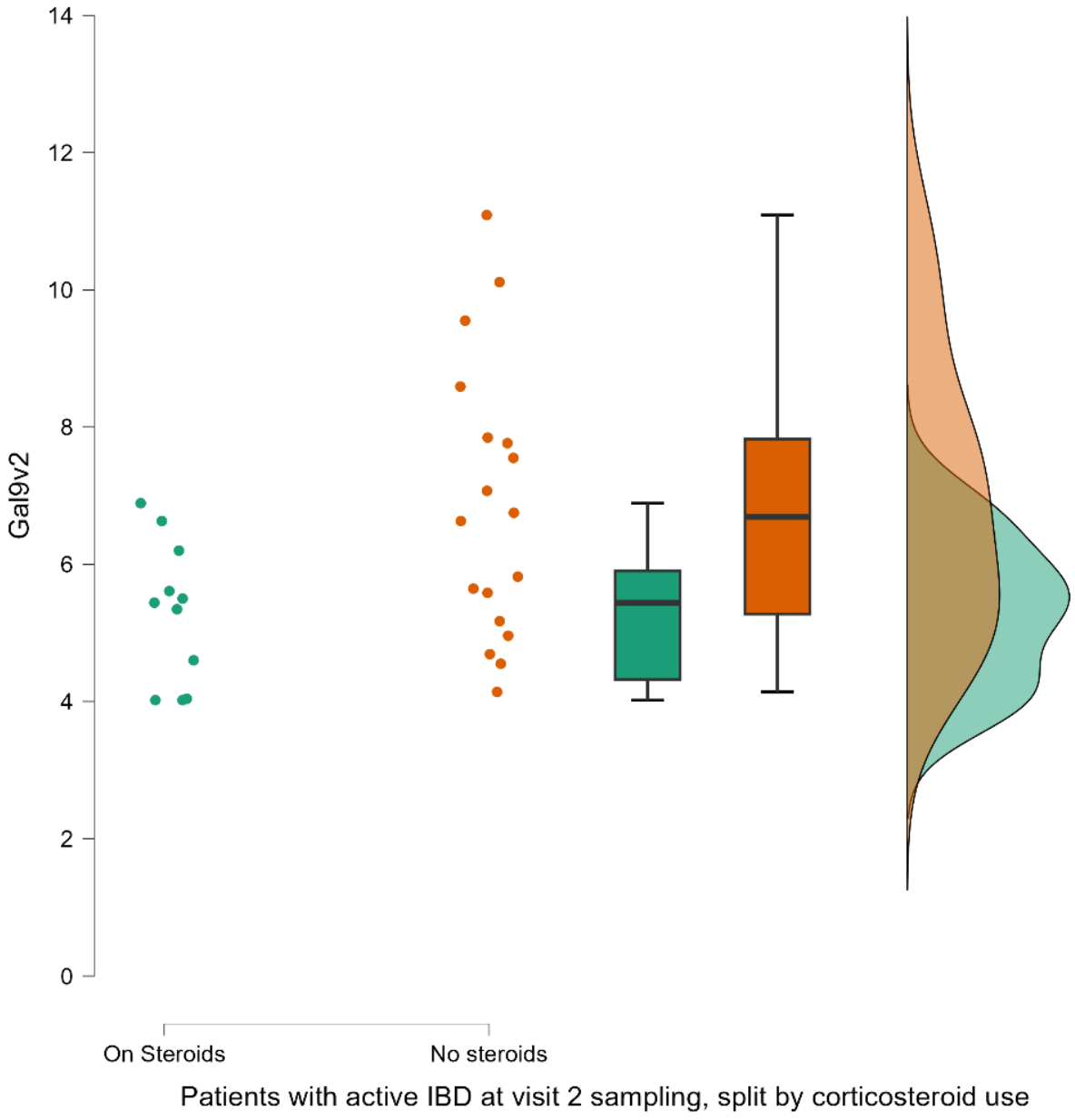


Figure 4 - 9: Marginal means generated from a regression model of GAL-9 level at visit 2, with disease state and corticosteroid utilisation.

Across the whole IBD cohort with a visit 2 GAL-9 result (n=57), a linear regression model (R2 0.149, F(2,46) 4.04 p 0.024) demonstrated that steroid use associated with significant reductions in GAL-9 level (t.234 p=0.024). In those with active disease at visit 2, corticosteroid use was associated with a lower median GAL-9 level (6.69 for those off steroids compared to 5.44 for those on steroids).

Inactive disease in these analyses implies meeting the pre-agreed criteria for remission. Early in the treatment course there may be several patients with a good treatment response moving towards remission that have not quite reached criteria yet. If the cohort is split as responders and non-responders, significantly higher GAL-9 levels are seen in non-responders (Mann-Whitney U 356 p=0.026). This however is a disease specific effect, with no difference between responders and non-responders in UC (Mann-Whitney U 37 p=0.89) and a highly significant change seen in CD (Mann-Whitney U 162.5 p=0.002). This is not seen with GAL-1 or -3.

Samples from a third time point were only available for 10 patients (CD=6 UC=4) which precluded generalisable conclusions being drawn. Nonetheless, separation between those with active disease and those reaching remission is seen in GAL-9 (Active n=7 median 7.54, Inactive n=3 median 5.41. Mann Whitney U = 1 p=0.04). Only one was patient still utilising corticosteroids at this timepoint. In the context of little steroid use, GAL-9 strongly associated with CRP (n=10 SR 0.803 p=0.009). The influence of AT on GAL-3 levels is less prominent in this small cohort that the larger pilot dataset. There is no significant difference in GAL-3 level between those on an AT or not at visit 3 (AT yes n=5 median 7.52 vs AT no n=5 median 7.42, Mann Whitney U 11 p=0.84). However, if you look at those who had been on that AT since visit 2 compared to all those that had not, allowing more time for the drug to reach maximal efficacy, significant differences are seen (AT V2 yes n=3 median 5.23 vs AT no n=7 median 7.8, Mann-Whitney U 0 p=0.017). Clearly, these signals need to be explored in a larger cohort before meaningful conclusions can be drawn.

4.4.6 Can baseline galectin level predict early response to treatment and future need for advanced therapies?

The analyses above show the associations between galectin levels and disease state across longitudinal sampling. However, they do not consider whether baseline levels can be utilised to predict early disease course, treatment responses and guide therapeutic choices. A current focus in IBD is the ability of apply predictive modelling to patients at presentation to allow early personalisation of care according to likely disease course. There is no currently available biochemical test used routinely in clinical practice can answer this question with any reliability. However, in our dataset, it is possible to demonstrate significant differences in baseline GAL-9 level in those who go on to require AT (within the first two years of follow up) and those that do not. This difference is explored in ***Figure 4-10.***

Differences can also be demonstrated in treatment response at set time points of disease assessment. Significant elevations in GAL-9 at baseline are seen to be associated with criteria defined non-response to treatment at 6 months on from diagnosis. This is particularly prominent in the small group of UC patients who did not respond to initial treatment with Mesalazine. These differences are explored in ***Figure 4-11***.

This significant difference is lost when treatment response is re-assessed at 12 months, though GAL-9 is still marginally higher in the cohort meeting criteria for non-response (n=109, non-response n=18 median 7.19, response n=91 median 6.28, Mann Whitney 936 p=0.34).

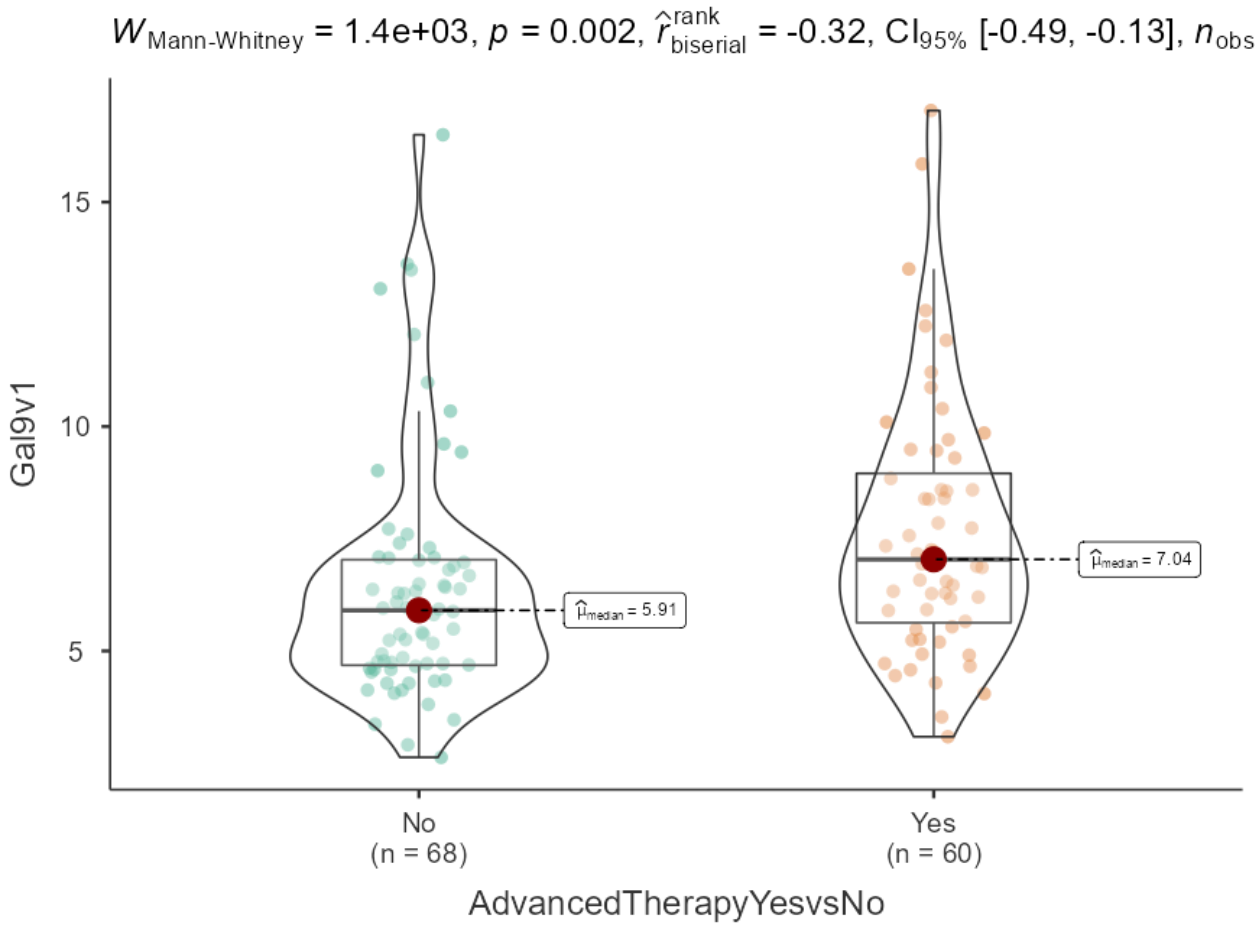


Figure 4 - 10: Median GAL-9 level at baseline pre-treatment, split by subsequent utilisation of AT during the first two years of IBD follow up.

This plot demonstrates findings across n=128 IBD patients (UC n=59 CD n=69) of whom 60 (UC n=16 CD n=44) have gone on to require AT during their first two years of follow up. The data demonstrates a statistically significant increase in baseline (pre-treatment) GAL-9 levels in those that go on to require AT (Median Yes = 7.04, median no = 5.91. Mann Whitney 1400 p=0.002). Whilst differences remain when these analyses are split into UC and CD is isolation, the statistical significance does not (UC median yes = 7.8, median no = 5.37, Mann Whitney 255 p=0.13. CD median yes = 6.97, median no = 6.37, Mann Whitney 451 p=0.22). No significant overall differences are shown in either GAL-1 (Mann-Whitney 2300 p=0.31) or GAL-3 (Mann Whitney 2100 p=0.76).

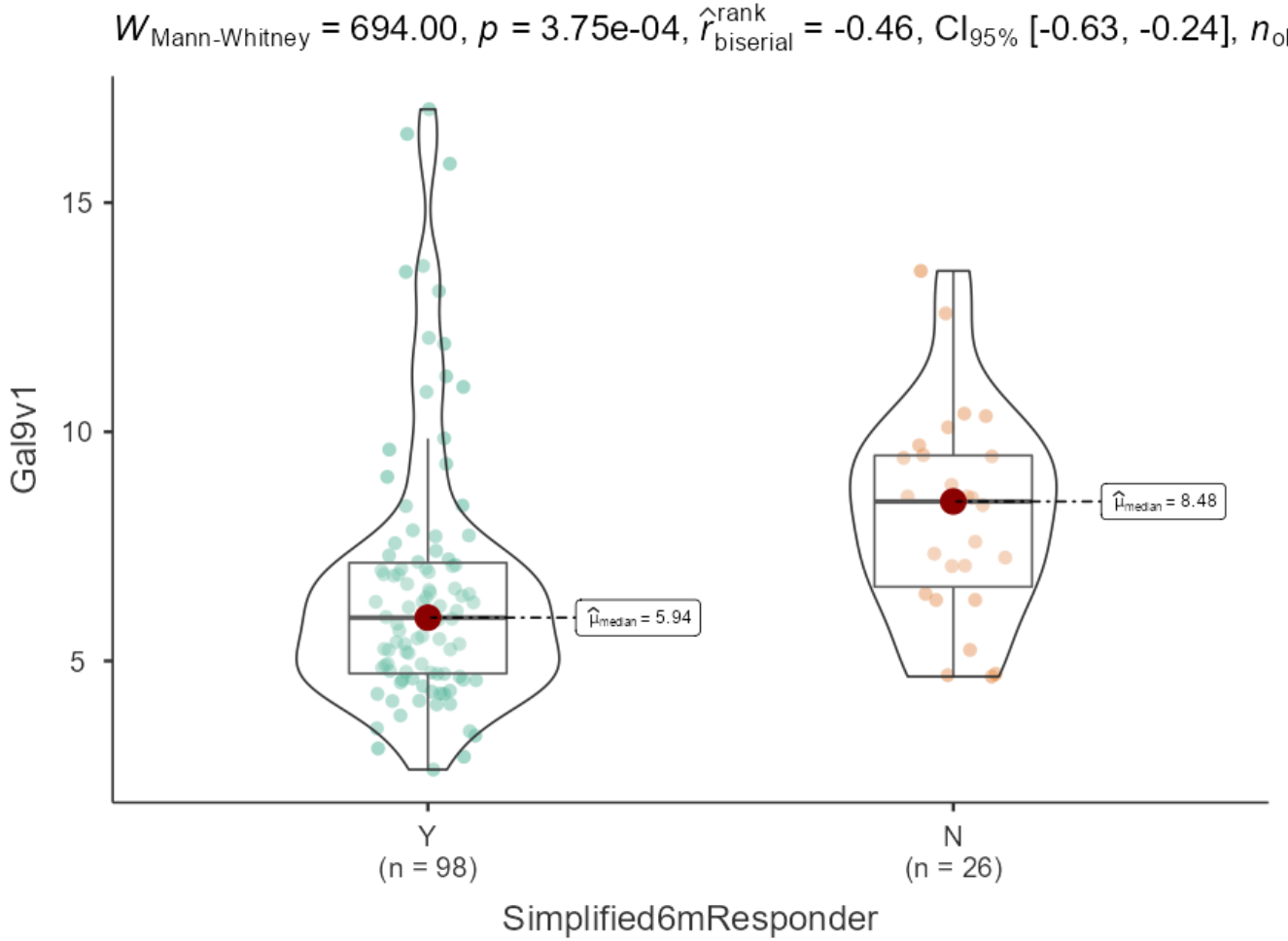


Figure 4 - 11: Median GAL-9 level at baseline, prior to treatment, split by treatment response at 6 months after diagnosis and treatment initiation.

This analysis was undertaken across a cohort of 124 IBD patients (CD n=66, UC n=58), of whom 26 (CD n=18, UC n=8) met criteria for 'non-response'. GAL-9 levels were significantly higher at baseline in those who subsequently did not respond to the first 6 months of standard medical treatment (all therapies), compared to those that did (Responder n=98 median 5.94, Non-responder n=26 median 8.48, Mann Whitney 694 p<0.001). This signal was stronger in UC (Responder n=50 median 5.26, non-responder n=8 median 9.04 p=0.003) than CD (Responder n =48 median 6.56, Non responder n=18 median 7.3 p=0.11). Again, this difference was in the overall cohort was not observed for either GAL-1 (Mann-Whitney 1100 p=0.30) or GAL-3 (Mann-Whitney 1200 p=0.80).

Considering the differences demonstrated relating to GAL-9, a regression model was developed to look for significant baseline predictors of treatment response and progression. A binomial regression model was established first for criteria-defined treatment response at 6 months. Covariates were GAL-9, Haemoglobin, CRP, Faecal Calprotectin, white cell count, platelet count, ferritin and age. IBD disk score was modelled as a covariate to provide a consistent clinical severity index across both disease subtypes. The model included 95 patients and demonstrated adequate fit (McFadden's pseudoR^2 = 0.28, overall model X^2 24.9 p<.001). The strongest predictor of non-response at 6 months was GAL-9 (Omnibus Likelihood ratio [LR] 10.23 p=0.001). Modelling GAL-9 alongside the top performing established serum biochemical indices (white cell count, ferritin and CRP) were taken into a machine learning decision tree classifier. The output from which is demonstrated in ***Figure 4-12***.

The initial regression model described above was also utilised for both 12-month treatment response and future need for AT. The strength of the model was substantially lower for 12-month response (n=86 McFadden's pseudoR^2 = 0.218, p=0.07) with no predictive capacity attributable to GAL-9. For AT, model fit was adequate (n= 97 McFadden's PseudoR^2 0.41, overall model X^2 55.1 p<.001). GAL-9 levels were just on the borderline of significance (LR 3.87, p=0.05). Both CRP (LR 11.03 p<.001) and Ferritin (LR 6.2 p=0.01) outperformed GAL-9 within the model.

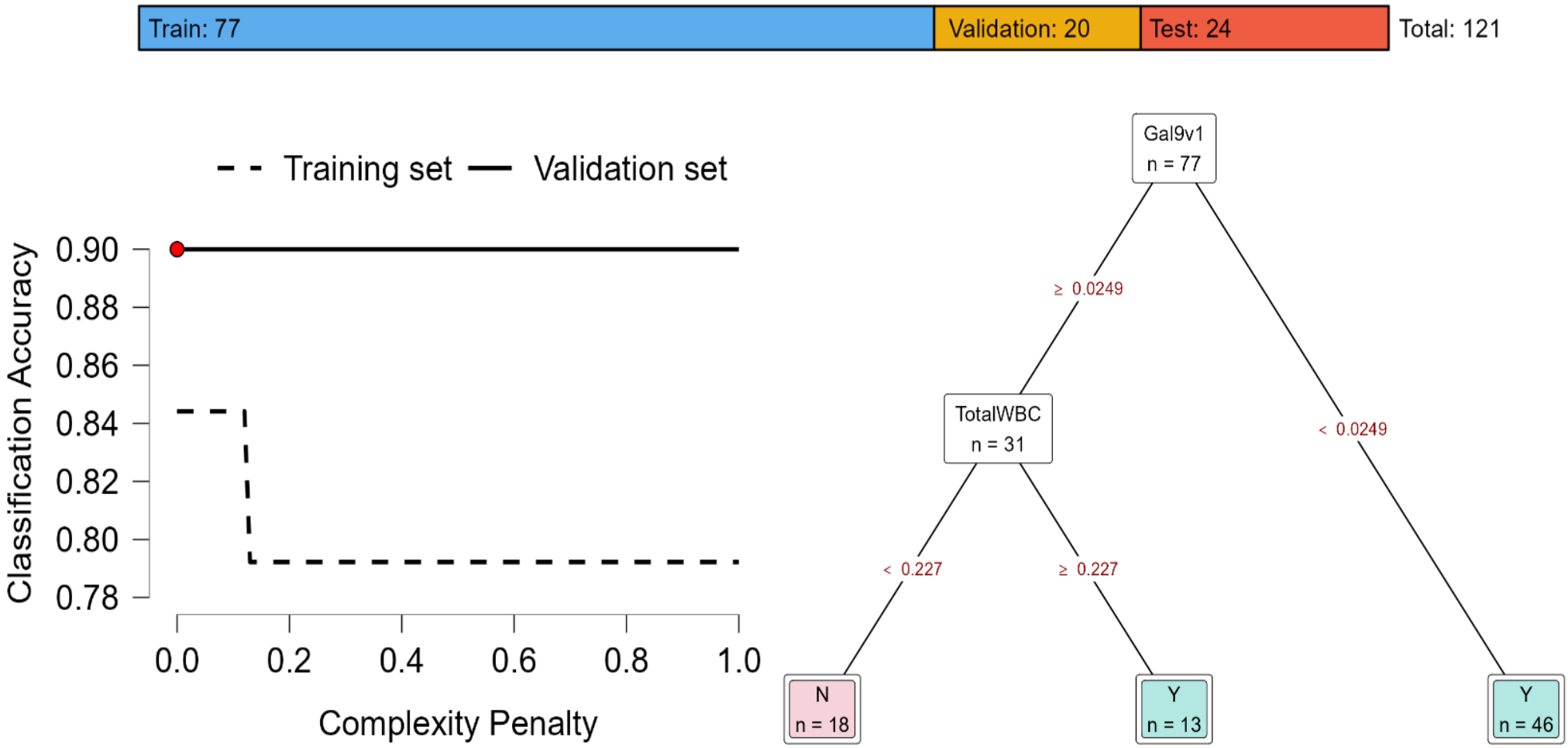


Figure 4 - 12: Machine learning decision tree (generated in JASP) utilising GAL-9, white cell count and ferritin to predict treatment response at 6 months

Using a random seed, the 121-patient cohort was split into training (77), validation (20) and testing (24) cohorts to look at ability to predict treatment response at 6 months. The testing cohort overall accuracy was 83.3% (Overall Positive predictive value 0.87, negative predictive value 0.91, AUC 0.71). GAL-9 levels were the most important variable within this model (relative importance 44.5, vs WBC 33.8, CRP 16.2 and Ferritin 5.5).

4.5 Discussion

I have presented two important cohorts in furthering our understanding of the relevance of galectins at IBD onset, allowing for the first time comparison between cohorts with established disease and prospectively collected longitudinal data from an inception cohort. This is the first cohort of its kind in the literature and only the second to look at any galectin in untreated IBD patients (Jovanovic et al., 2019). Through the ability to compare levels pre and post treatment, it is possible to quantify the impacts of specific therapies. By tracking outcomes longitudinally, it is possible to interrogate the potential role for galectins as a biomarker in early IBD.

4.5.1 Comparing the pilot and inception cohorts

The pilot dataset and the inception cohort share several similarities, but there are several key differences. Both datasets demonstrate significant increases between GAL-3 and GAL-9 in IBD (across subtypes) relative to HC. Changes in GAL-1 are not significant in either cohort. In the pilot cohort, very few associations are seen with disease severity indices or biochemical parameters. However, within the inception cohort, GAL-9 significantly correlated with other disease activity indices. Despite not showing significant differences longitudinally, GAL-1 demonstrated weak associations that more closely align with mucosal disease activity, including FCP. For CD, GAL-1 correlated with both endoscopic and clinical disease severity.

Some of the post treatment signals, for example the lower GAL-3 levels in patients on biologic therapy within the pilot cohort, are not currently replicable in our limited inception cohort with longitudinal sampling. The additional granularity to the

associated metadata available with the inception dataset was also able to highlight the significant association between corticosteroid use and reductions in GAL-9 level even in those with persisting disease activity.

4.5.2 Comparing our outputs with existing understanding

Several of these novel relationships with clinical metadata tie in with what has been described in the literature, though not all of them result in a clearer picture. Papa-Gobbi et al. (2016, 2021) repeatedly demonstrated increased expression of GAL-1 mRNA in the colonic mucosa in areas of severe inflammation. Neither of these papers looked at the relationship between mucosal levels of GAL-1 and how these translate to what is seen in the serum. Kessel et al. (2021) did look at serum and showed higher GAL-1 levels were seen in those with unstable remission. Though our data would agree that GAL-1 is associated with mucosal inflammation, almost the opposite relationship has been shown, with GAL-1 negatively associated with both FCP and SES-CD.

GAL-3 is perhaps the most studied of the galectins in IBD. Both Volarevic et al. (2019) and Jovanovic et al. (2019) demonstrated that though GAL-3 levels were higher in UC than HC, the highest GAL-3 levels were seen in those with the mildest disease. In our UC cohort, the highest GAL-3 level was seen in those with Mayo 1 disease (8.21 vs Mayo 2 7.04, Mayo 3 7.72), though none of these differences was significant using a Kruskal-Wallis or pairwise comparisons (overall p=0.255). Neither of these prior studies included patients with CD. In our CD cohort the pattern is not seen, with the highest GAL-3 levels in those with SES-CD scores falling into the

severe range at diagnosis (9.28 vs mild 8.17 and moderate 7.81, no global or pairwise significant difference [KW (2) 1.27 p=0.53]). The presence of lower GAL-3 levels in patients established on biologic therapy, irrespective of inflammatory activity is one of interest. I could not find this reported in an IBD setting. It is known however that the crosstalk between cytokines and galectins is complex. Both Jensen-Jarolim et al. (2002) and Muller et al. (2006) showed that stimulation with TNF resulted in GAL-3 downregulation. It does not necessarily follow then that administration of AT (the majority of which were anti-TNF) should result in reduced extra-cellular GAL-3 levels. There are many cytokine – galectin interactions however and these are known to be complex. TNF-α has been shown to induce GAL-9 secretion in vitro from mesenchymal stem cells, whilst the sustained presence of IL-10 has been shown to increase GAL-3 binding (Górdon-Alonso et al., 2018).

GAL-9 has to date been relatively under explored in IBD. Differences in mucosal expression have been demonstrated in several studies (Shi et al., 2012; Papa-Gobbi et al., 2016) but the one study analysing serum levels in a mixed cohort found no difference (Cibor et al., 2016). The work by Chen et al. (2020), whilst a secondary analysis, is an important association with our work given that they were able to demonstrate enrichment of LSGALS9 transcripts in both mucosa and PBMCs from patients with new onset CD. Within our cohorts, GAL-9 is related to disease activity and systemic inflammation, particularly in CD.

4.5.3 Are galectins are viable biomarker for IBD diagnosis?

GAL-3 and -9 are effective at distinguishing IBD from HC across both the pilot and inception cohorts. However, in symptomatic patients referred with a transiently

elevated FCP and suspected IBD but subsequently normal investigations (18 diagnosed with IBS, 3 haemorrhoids), there was no difference between any of the galectins measured when compared with IBD subtypes.

It is clear from this small cohort that we are not imminently going to be applying the measured galectins as a serum version of the faecal calprotectin to identify IBD. I believe however that our longitudinal outcome data does show where the clinical utility of serum GAL-9 levels might make it a valuable part of the work up of patients with suspected IBD.

4.5.4 Galectin-9 as a predictor of early disease therapeutic outcomes: a valid measure to aid prognostication?

In current IBD paradigms, personalised medicine is the goal. In its most basic form, it relates to our ability understand an individual patient's disease biology to the extent of being able to predict their future disease course from the outset. The aspiration is not just to be able to identify the need for aggressive treatment, but also which treatment they are likely to respond to. This will involve a deeper understanding of individual genetic risk profiles, microbial community structure, host-immune interactions and metabolomic factors. It would be overblown to suggest GAL-9 as an answer to this question. Our data would suggest however it could form part of that process. In a regression model of treatment response at 6 months, GAL-9 outperformed all other readily available biochemical indices including FCP. It was a significant predictor of non-response to treatment. In a machine learning decision tree model, it was the strongest performing predictor in a model of biochemical indices that carried an overall accuracy of 90% in its validation and 83% in its testing

cohorts. Whilst it did not demonstrate an ability to discriminate response at 12 months, GAL-9 levels were elevated in those going to AT. Whilst these findings would need to be validated in a larger multi-centre study, if proven, they would undoubtedly add to our ability to stratify patients at IBD onset, across disease subtypes. After presenting the Microbiome data in chapter 5, I will explore whether integration of these datasets can increase predictive value.

4.6 Conclusion

For the first time at IBD onset, our study has demonstrated significant increases in serum GAL-9 that correlate with systemic markers of inflammation. GAL-1 levels show also correlate with levels of mucosal inflammation at CD onset. GAL-9 level at baseline is elevated in those progressing to AT and can be utilised to help predict early treatment responses. We have demonstrated novel reductions associated with corticosteroid use and shown enduring associations with inflammatory activity over longitudinal follow up, albeit in a small cohort. The potential role of GAL-9 as a predictor of early disease treatment outcome warrants exploration in further multicentre studies.

CHAPTER 5: Describing the pre-treatment gut microbiome at IBD onset

5.1 Abstract

Introduction

Disruption of the gastrointestinal microbiome is a recognised feature of IBD. Studying newly diagnosed pre-treatment patients offers the greatest insight into how microbial factors contribute to disease onset. Existing studies focus on paediatric populations, with limited adult data. Methodological variation contributes to conflicting results. We present the analysis of the faecal microbiota from a large adult pre-treatment IBD cohort and integrate this with published raw sequence datasets.

Methods

For the Birmingham cohort, stool collected in DNA Genotek OM-200 kits was brought to the first pre-diagnosis outpatient review. Clinical indices were collected longitudinally. Stool microbial DNA was extracted. 16S rRNA PCR and sequencing was undertaken. 16S diversity analyses and taxonomic classifications were performed in line with the QIIME2 workflow. Shotgun metagenomics was performed for a sub-group of IBD patients and analysed using MetaPhlAn4, HUMAnN 3 and the Vegan plugin for R. Taxa associations were determined using MaAsLin2 and abundance changes expressed as L2FC. A FDR of <0.2 was considered relevant, with <0.05 highly significant.

The 16S rRNA data was then integrated with available and author-shared pre-treatment raw sequence datasets from publications identified in our earlier

systematic review. A bioinformatic pipeline was repeated, again in line with the QIIME2 workflow. Here, differential abundance was assessed using DESeq2.

Results

In the Birmingham dataset 93 IBD patients (53 CD, 40 UC) were sequenced alongside 52 SC. The shotgun dataset included 45 CD and 33 UC patients. Alpha diversity was not significantly different between IBD and SC, but IBD patients with lower baseline diversity were more likely to subsequently progress to AT in both the 16S and shotgun dataset. In the latter, this was significant for both CD (median Shannon index 3.02 vs 2.34 p=0.018) and UC (2.66 vs 1.57 p=0.009). In the 16S data, IBD was separated from SC by enrichment of phenotypically oral bacteria including *Fusobacterium, Haemophilus and Peptostreptococcus.* There was depletion of core SCFA producers including *Oscillibacter* and *Moryella* (all FDR <0.2). In UC, patients who failed to sustain responses to steroid induction and mesalazine therapy, enrichment of *Fusobacterium* and *Haemophilus* was striking (FDR <0.01). Enrichment of *Peptostreptococcus, Veillonella* and *Aggregatibacter* was also observed (FDR <0.2). Depletion was characterised to strain level in the shotgun data, with significant reduction of strains of *Fusicatenibacter saccharivorans, Bifidobacterium longum* and *adolescentis* concordant with the 16S data. Across grouped IBD patients requiring AT, enrichment and increased metabolic activity of *Sutterella wadsworthensis* was observed.

These findings were integrated with a pooled dataset ultimately totalling 18 studies and 2160 samples (1513 IBD, 647 controls). Overall, mucosal biopsies displayed

lower alpha diversity than stool (Shannon index; faeces median 2.47, biopsy median 2.06 p<.001). The closest association in beta diversity between sample types was observed in UC. Diverse patterns of difference in alpha diversity were observed across sample types and patient age groups. For example, faecal alpha diversity was significantly reduced in paediatric UC vs SC but increased vs SC in mucosal biopsies. Enrichment of bacteria typical of the oral cavity including *Fusobacterium, Haemophilus, Aggregatibacter* and *Porphyromonas* was observed across multiple sample types and comparisons.

Conclusion

Reduction in alpha diversity at IBD onset is not consistent across sample types and age groups, but baseline reductions are linked to the failure of conventional therapies and the need for advanced treatments. An enrichment of bacteria typical of the oral cavity is typical in both CD and UC onset and within the Birmingham data is linked to increased disease severity and a failure of conventional IBD therapies.

5.2 Introduction

The current literature relating to the gut microbiome at IBD onset has been explored at length in section 1.3. Certain core certain themes emerge from Inception IBD datasets, though deficiencies in the data, such as a lack of methodological standardisation and a paucity of data from adults represent key limitations. Given the significance of microbiome disruption at IBD onset, there is a need for further characterisation. Through this chapter I will present analyses of our dataset, followed by an integration of our data with publicly available or author shared raw sequence datasets. The methodologies applied to our dataset are described in chapter 2, though the methodology for the integrated analysis is discussed separately during this chapter given its complexity. Raw bioinformatic outputs meeting statistical significance are contained within the appendices.

5.3 Results

5.3.1 The Birmingham IBD inception 16S rRNA sequencing cohort

An initial 199 samples were sequenced after PCR amplifying the V4 region of 16S rRNA gene. Two samples were lost during rarefaction. These had achieved poor amplification during the PCR and their loss taken to represent a primer problem. From the available samples, a mean read frequency of 45 079 reads per sample were obtained. Samples reads were trimmed above the highest negative control (5892 reads), resulting in the further loss of 3 samples and the lowest read count retained being 11193 reads (***Figure 5-1***).

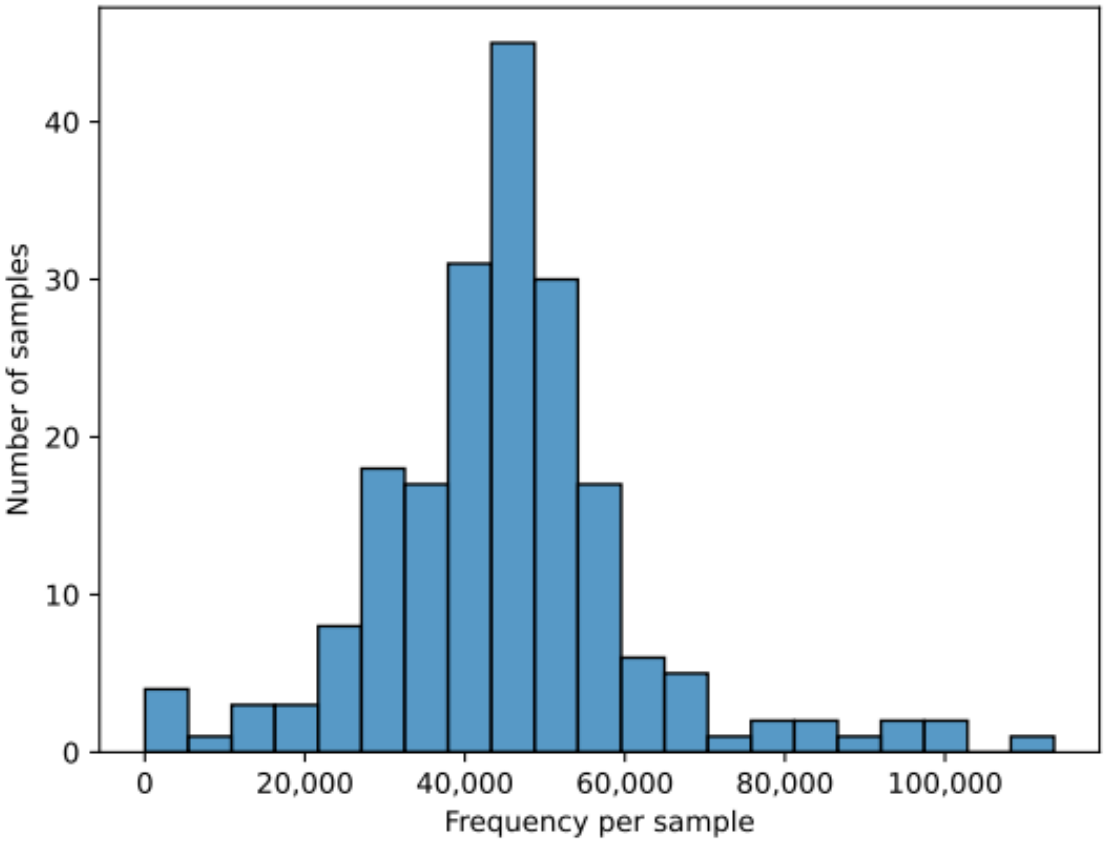


Figure 5 - 1: Distribution of read counts obtained from 16S rRNA sequencing of the V4 domain.

Most samples clustered above 40000 reads per sample. Using the DADA2 workflow, reads were trimmed above the lowest count obtained from negative controls, resulting in the loss of three samples.

Baseline pre-treatment samples from an initial 151 patients were retained in the dataset. 93 had IBD and 58 were SC. Upon first analysis no significant differences were seen in alpha diversity between CD or UC and controls (Shannon entropy, Kruskal-Wallis; pairwise CD 0.77 p=0.38 UC 0.46 p=0.49). Significant differences were seen however with beta diversity. Using the Bray-Curtis distance, significant differences were seen using pairwise PERMANOVA between SC and both UC (n=117, permutations 999, pseudo 1.864 p=0.008 q=0.012) and CD (n=131, permutations 999, pseudoF 2.085 p=0.004 q=0.012). There was no difference between IBD subtypes (n=124, permutations 999, pseudoF 1.092 p=0.31 q=0.31).

However, the SC cohort as it started included some serious organic pathology. Those with clinically relevant diagnoses were then excluded as below:

- 2 subsequently diagnosed with colorectal cancer.
- 1 with HIV enteropathy, giardia and rectal chlamydia
- 1 with ischaemic colitis
- 1 with microscopic colitis
- 1 in whom investigations were continuing at the time of analysis.

After exclusion, the final cohorts utilised for the presented analyses are shown in ***Table 5-1***. Cohorts were well matched in age (Kruskal-Wallis [2] 1.88 p=0.39), with differences in sex not reaching significance (X^2 5.26 p=0.07). Antibiotic usage in the 28 days prior to sampling was higher in SC but population wide differences did not reach significance (X^2 7.95 p=0.09).

Table 5 - 1: Patient demographics and disease distribution: IBD and composite control cohort 16S rRNA sequencing

	Crohn's **n = 53**	UC **n = 40**	Symptomatic controls **n = 52**
Age, median(IQR), y	34 (20)	36 (15.8)	35 (19.3)
Sex, n (%) male	18 (34)	23 (58)	21 (40)
Ethnicity, n (%)			
Asian	9 (17)	7 (17.5)	8 (15)
Black	4 (9)	3 (7.5)	4 (8)
White	40 (75)	30 (75)	40 (77)
Number utilising antibiotics in the month prior to sampling, n (%)	3 (3.7)	2 (5)	10 (19)
Baseline FCP, median (IQR), ug/g	676 (1491)	1489 (1640)	557 (704)
FCP at time of 16S sample, median (IQR), ug/g	568 (836)	1031 (1987)	62 (144)
Disease distribution / Non IBD diagnosis, n (%)	Ileal: 26 (49) Colonic: 11 (21) Ileocolonic: 16 (30)	Proctitis: 10 (25) Left sided: 12 (30) Extensive: 18 (45)	Functional symptoms = 34 (65) Resolved infection = 8 (15) Misc (e.g., haemorrhoids, gallstones) = 10 (19)
Subsequent biologic utilisation, n (%)	Yes: 31 (58) No: 22(42)	Yes: 9 (22.5) No: 31 (77.5)	

5.3.1.1 Comparing the cohorts

When re-comparing IBD and SC, the observed differences in Shannon alpha diversity did not reach statistical significance ***(Figure 5-2)***. This was true of grouped IBD and IBD split by subtype.

Differential microbial abundance according to diagnosis was then interrogated ***(Figure 5-3)***. The group IBD cohort was characterised by a depletion of multiple firmicutes, including genus level depletion (FDR <0.2) of *Moryella, Catenibacterium, Phascolarctobacterium, Holdemanella* and *Eubacterium coprostanoligenes.* Alongside this, there was a significant increase in abundance of typically oral pathobionts such as *Fusobacterium* and *Haemophilus.* However, when the comparisons are split by IBD subtype, a diverging patterns are observed. UC is almost entirely responsible for the overall increases in abundance of *Haemophilus, Fusobacterium, Veillonellaeceae* and *Peptostreptococcocus* with an added increase in *Gemella.* In CD, depletion of *Firmicutes* is seen again, with significant enrichment of a single *cyanobacteria.*

Comparing CD and UC, the only significant difference identified was greater depletion of Bifidobacterium in CD (L2FC 1.9 p 0.001 FDR 0.14)

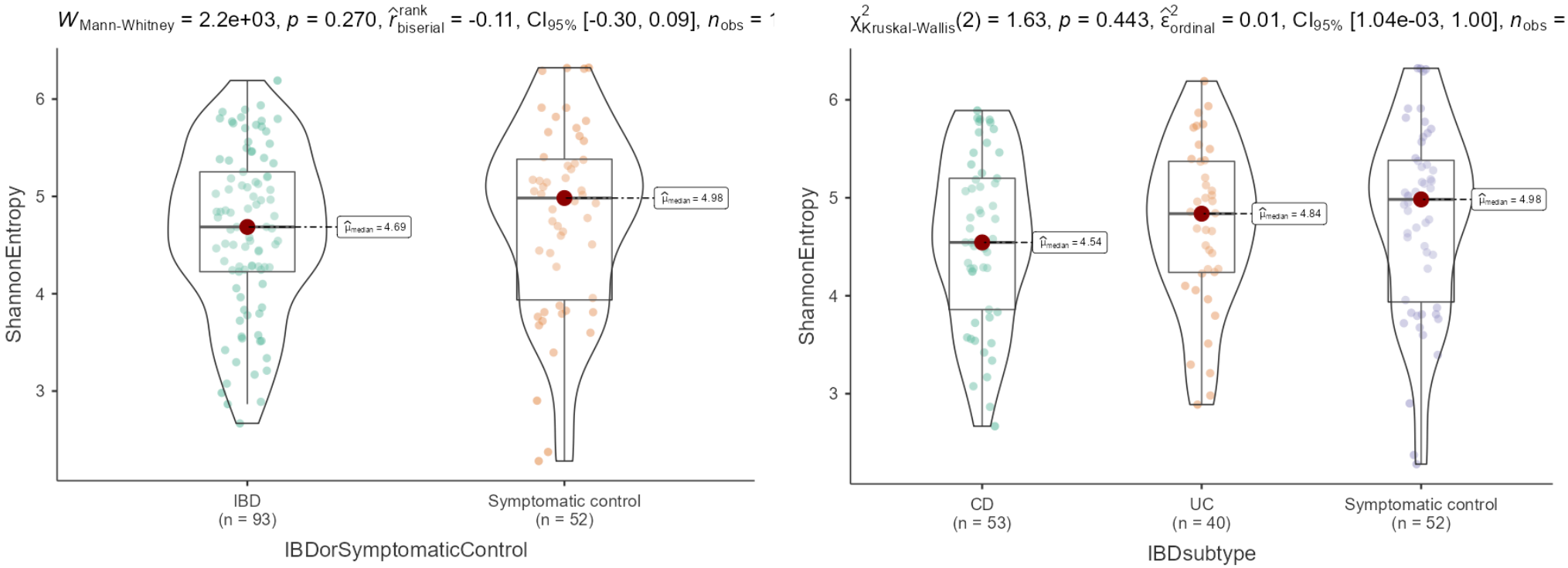
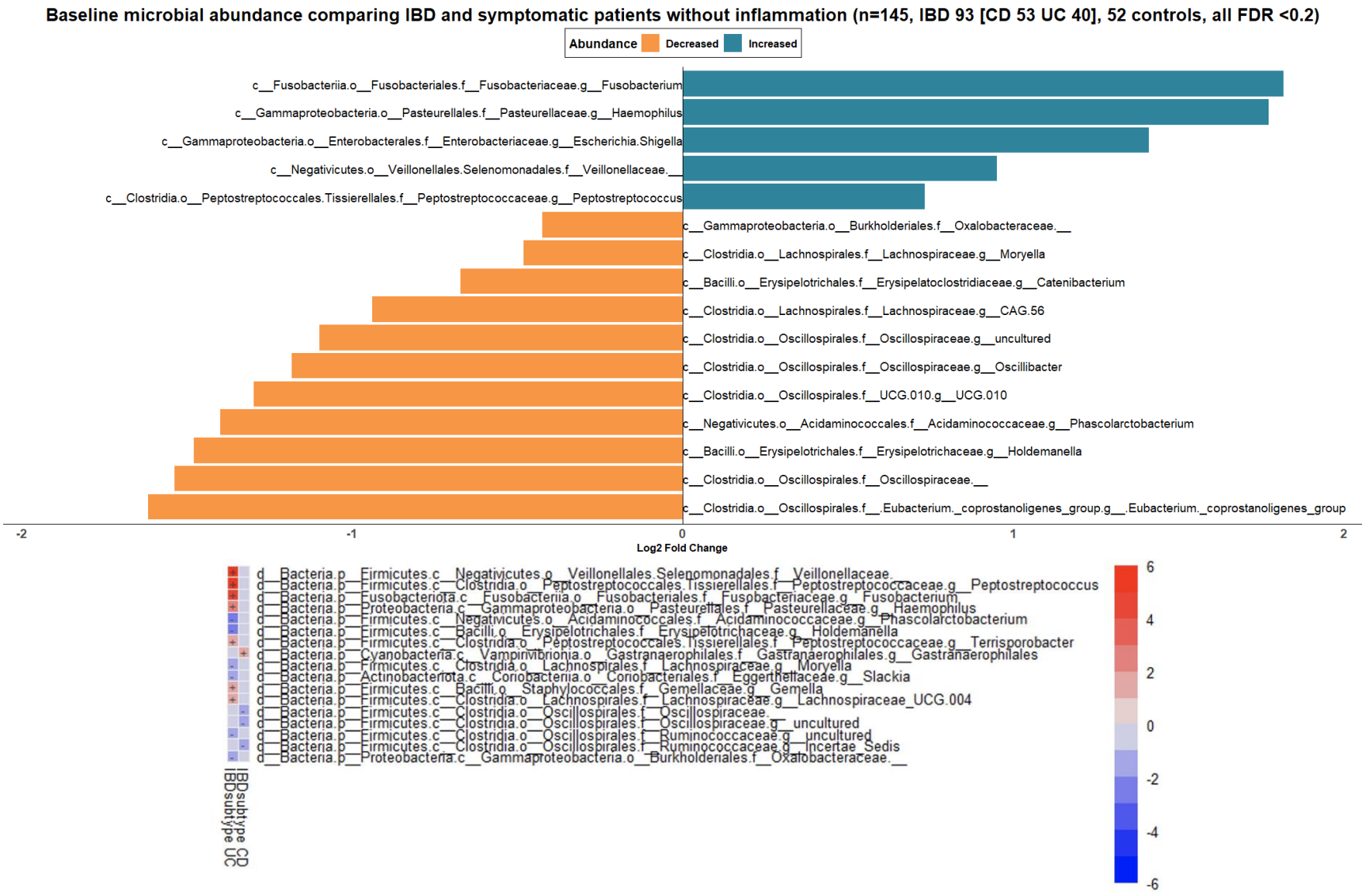


Figure 5 - 2: Comparing alpha diversity (Shannon Entropy) amongst patients with IBD (both as a whole and split by subtype) and symptomatic controls
These two plots demonstrate that whilst median alpha diversity may be marginally lower in inflammatory bowel disease, this does not reach statistical significance within our dataset. This is true of grouped IBD (IBD n = 93 median 4.69 vs symptomatic control n = 52 median 4.98, Mann-Whitney 2200 p = 0.27) or when IBD is split by subtype (CD n =53 median 4.54 UC n = 40 median = 4.84 symptomatic control n = 52 median 4.98, Kruskal-Wallis (2) 1.63 p=0.44).



*F*igure 5 - 3: Differential abundance of key taxa between IBD and controls

As described above, there are several significant differences between the two cohorts (n=145). These differences follow distinct patterns between CD and UC. UC is characterised by increases in abundance of oral type pathobionts. At genus level, this includes *Haemophilus* (L2FC -2.43 FDR = 0.06*), Fusobacterium* (L2FC -2.85 FDR = 0.01) and *Peptostreptococcus* (L2FC -1.39 FDR 0.006). At family level*, Veillonellaceae* is significantly enriched (L2FC 1.50 FDR = 0.006). Less marked signals are seen isolated to CD, with enrichment of *Gastranaerophilales* (L2FC 1.08 FDR 0.17) and depletion *of Oscillospiraceae* (L2FC -1.54 FDR 0.19).

5.3.1.2 Topic Analysis of the Microbiome at IBD onset

For patients with CD, six different topics were identified with significant separation from SC. Only two of these maintained a $p<0.05$ when corrected for multiple testing, though five remained <0.1. Two highlighted the marked increase in abundance of *Enterobacteriaceae*, particularly *Escherichia-Shigella* in patients with CD. Relative increases in the abundance of *Veillonella*, *Haemophilus* and *Sutterella we*re also seen. Interestingly, a final topic highlighted an increase in *Akkermansia* in CD relative to SC. The same process was utilised to contrast UC and SC. Four topics showed significant separation, but only one had an adjusted p-value <0.05 (or 0.1). In addition to replicating the significant signals seen earlier regarding *Haemophilus* and *Fusobacterium,* small increases in *Escherichia-Shigella, Ruminococcus Gnavus, Campylobacter and Streptococcus* were identified*.* Significant depletion of *Prevotella, Agathobacter,* and *Eubacterium Coprostanoligenes* were observed alongside smaller reductions in *Roseburia* and *Faecalibacterium.* Between CD and UC, three significant topics were seen, with one maintaining a $p<0.05$ after correction, the other staying $p_{adj}<0.1$. The same significant depletion of *Bifidobacterium* in CD was identified, alongside marked reductions in *Faecalibacterium, Eubacterium Eligens* and *Coprococcus* relative to UC. ***Figure 5 - 4*** presents three of these Topics, one for each comparison, with each retaining a $p_{adj}<0.05$.

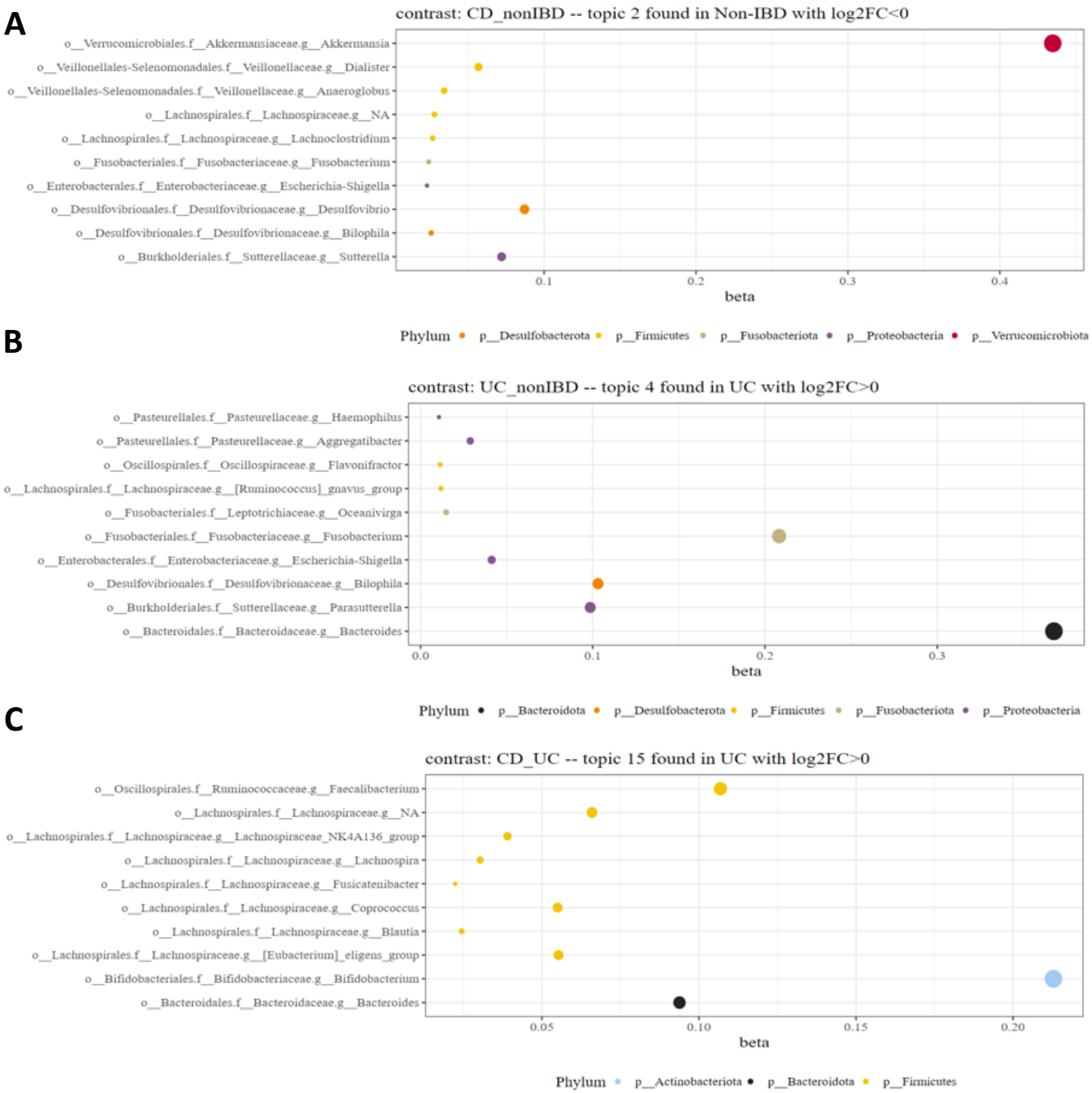


Figure 5 - 4: Selected Topic analyses of the IBD Inception Microbiome

A. Topic 2 contrasting CD vs SC highlights enrichment of Akkermansia, a taxon often associated with health, alongside pathobionts such as *Escherichia, Shigella* and *Fusobacterium.* The overall grouped L2FC for this Topic was -2.816 (standard error 1.02, p-adjusted 0.045).

B. The contrast next is between UC and SC. Topic 4 highlights the enrichment of key pathobionts in UC such as *Fusobacterium, Bilophila, Escherichia, Shigella and Ruminococcus Gnavus,* but also highlights significant enrichment of *Bacteroides* in UC patients. The overall grouped L2FC for this topic was 3.737 (standard error 1.172, p-adjusted 0.027).

C. The final comparison is between CD and UC. Topic 15 highlights the greater depletion of core SCFA producers such as *Bifidobacterium* and *Faecalibacterium* in CD. The overall grouped L2FC for this Topic was 3.197 (Standard error 0.99, p-adjusted 0.024).

5.3.1.3 Microbial associations with disease severity at IBD onset

It is possible to demonstrate significant associations between clinical measures of disease severity and alpha diversity (Shannon) in both CD and UC. This is not possible however for endoscopic severity. In CD, HBI carries a significant association with Alpha Diversity (r_s= -0.409 p=0.003), as does Partial Mayo in UC (r_s = -0.417 p 0.007). This is shown in ***Figure 5-5.***

5.3.1.3.1 Montreal disease extent, location and behaviour

It was not possible to identify any significant microbial differences in CD when comparing disease location or behaviour. In UC significant reductions in abundance of two Firmicutes, an uncertain *Ruminococcus (Incertae Sedis* L2FC E1vsE3 -3.1 FDR 0.056) and *Erysipelotrichaceae (UCG* L2FC E1vsE2 -2.95 FDR 0.056) were observed in more extensive disease. Depletion of *Collinsella* (L2FC E1vsE3 -2.58 FDR 0.58) was also observed with increasing extent. Similar reductions in *Bifidobacterium* sat outside of statistical significance (FDR 0.24).

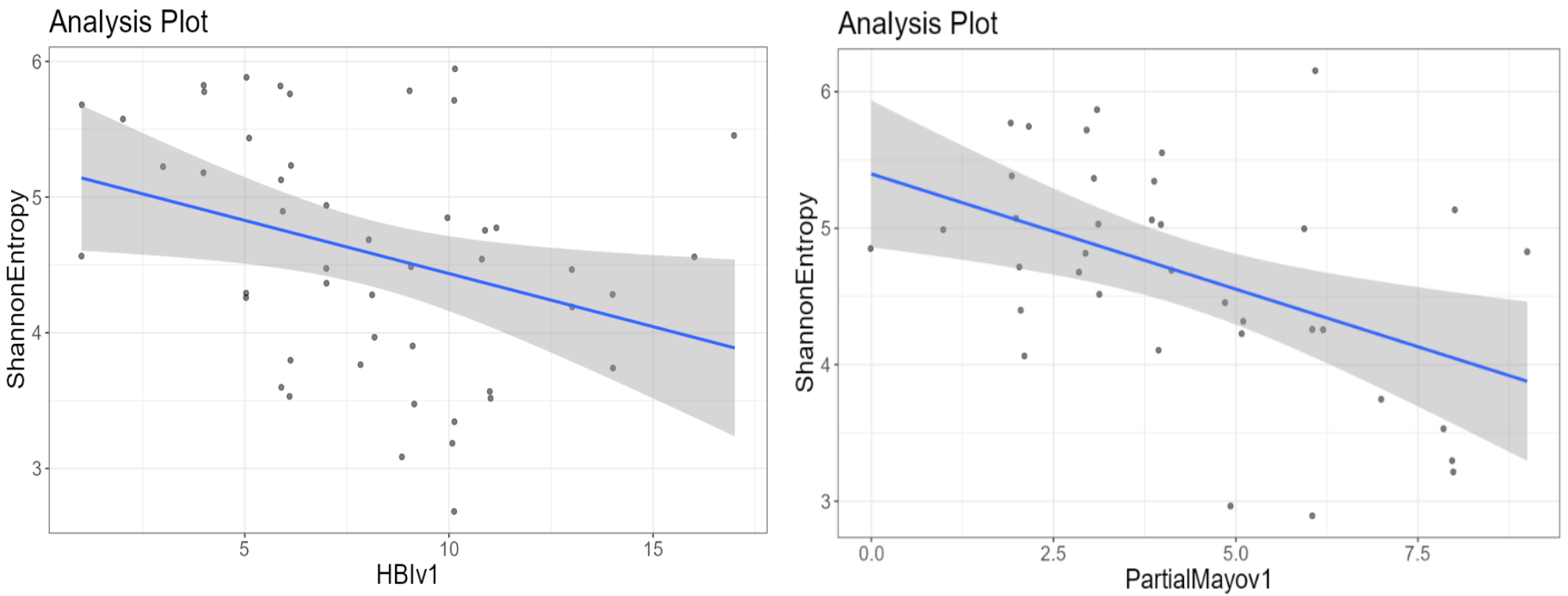


Figure 5 - 5: Associations between alpha diversity and clinical disease severity at IBD onset.

These scatter plots demonstrate the correlation between clinical severity indices in both UC and CD, at disease onset. In CD (n=50), Shannon entropy significantly correlated with HBI (Spearman's Rho r_s= -0.409 p=0.003). In UC (n=40), Shannon also correlated with Partial Mayo score (r_s= -0.417 p=0.007). Three patients in the CD cohort had missing data for HBI, and hence are excluded from this analysis.

5.3.1.3.2 Microbial associations with Faecal Calprotectin level

Across the cohort, including SC, multiple strong associations are seen with FCP level (composite), as shown in ***Figure 5-6***. Marked increases in *Fusobacterium* (FDR <0.001), *Gemella* (FDR 0.003), *Peptostreptococcus* (FDR 0.004), *Porphyromonas* (FDR 0.03) and *Haemophilus* (FDR 0.06) are seen to associate with higher FCP levels, whilst known SCFA producers including *Oscillibacter* (FDR 0.05), *Oscillospira* (FDR 0.06) and *Phascolarctobacterium* (FDR 0.19) are. Interestingly, these associations are weaker when the IBD cohort is evaluated in isolation, though significant increases in abundance of *Fusobacterium* (FDR 0.01) and *Gemella* (FDR 0.01) continue to strongly associate with a higher FCP.

5.3.1.3.3 Clinical and Endoscopic severity in UC

In UC, significant associations were identified between Partial Mayo score and 48 different taxa. The strongest associations (FDR <0.05) were for depletion of several *Eubacteria (Ventriosum, Coprostanoligenes and Halli),* alongside an unclassified *Ruminococcus (Incertae Sedis)* and increasing Partial Mayo scores. Increases in abundance of *Veillonellaceae* (FDR 0.03) and proteobacteria including *Aggregatibacter* (FDR 0.058), *Haemophilus* (FDR 0.07) and *Fusobacterium* (FDR 0.09) were again prominently associated with higher partial mayo scores.

Increased abundance of *Fusobacterium*, *Veillonellaceae and Escherichia.Shigella* also correlated with increased UCEIS, alongside depletion of *Bifidobacterium, Ruminococcus Gauvreauii and Agathobacter* (all FDR 0.17 – 0.19).

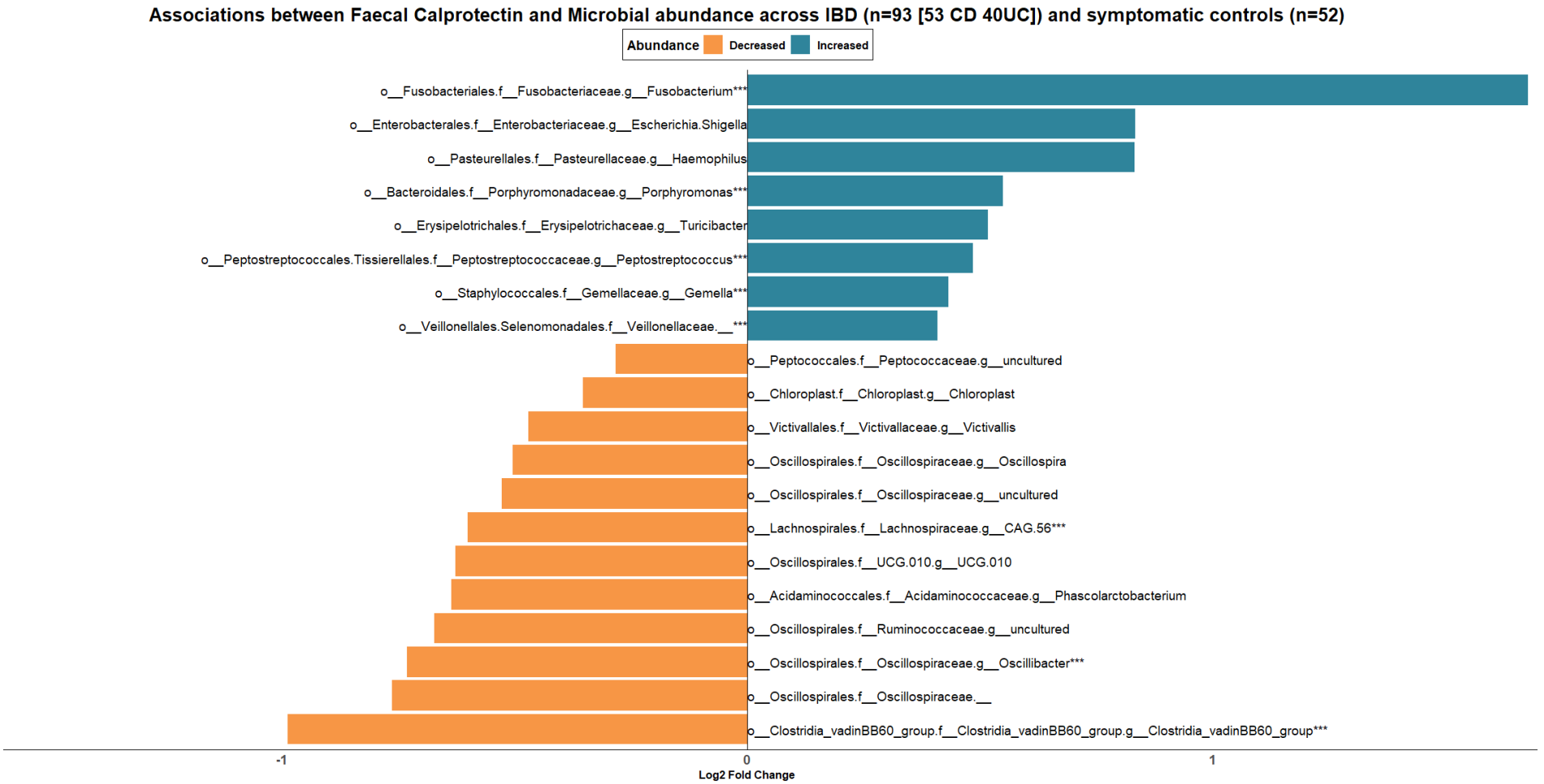


Figure 5 - 6: Differential abundance seen with FCP across our entire cohort, encompassing 93 IBD patients and 52 symptomatic controls.

Associations with a false discovery rate of less than 0.05 included positive associations with *Fusobacterium* (L2FC 1.7 FDR <0.001), *Gemella* (L2FC 0.4 FDR 0.003), *Peptostreptococcus* (L2FC 0.5 FDR 0.004) and *Porphyromonas* (L2FC 0.05 FDR 0.03) and negative associations with *Clostridia Vadin BB60* (L2FC -0.98 FDR 0.04) and *Oscillibacter* (L2FC -0.7 FDR 0.05). When evaluating IBD patients in isolation, significant positive correlations remain with *Fusobacterium* (L2FC 1.57 FDR 0.01) and *Gemella* L2FC 0.57 FDR 0.01).

5.3.1.3.4 Clinical and Endoscopic Severity in CD

Fewer meaningful associations were observed. For HBI, no significant correlations were identified. For endoscopic severity (SESCD), depletion of *Eubacterium Eligens* (L2FC -0.8 FDR 0.04), *Roseburia* (L2FC -1.5 FDR 0.1) and enrichment of *Erysipelatoclostridium* (L2FC 0.46 FDR 0.04) were observed with increased severity.

5.3.1.4 Microbial associations with overall disability in IBD

Our dataset has allowed the connection between the gut microbiome and overall disability at IBD onset (IBD disk). These associations were specific to IBD, with none maintained when scores from IBD and SC were grouped for analysis. Alpha diversity (Shannon) associated both with total disk score (r_s = -0.266 p 0.025) and 'Emotions' domain scores (r_s = -0.270 p 0.024). Increased abundance of *Lachnoclostridium* was strongly associated with increased total disk score (FDR 0.01), whilst those with higher disability demonstrated depletion of *Lactobacillus* (L2FC -0.68 FDR 0.09), *Faecalibacterium* (L2FC -0.84 FDR 0.16), *Agathobacter* (L2FC -1.0 FDR 0.16) and *Fusicatenibacter* (L2FC -0,71 FDR 0.16). When correcting for mucosal inflammation using the composite FCP, all these associations, bar *Fusicatenibacter*, maintain an FDR of <0.2, with *Lachnoclostridium* remaining <0.05. Abundance of *Lachnoclostridium* also correlated positively fatigue scores (*'Energy'* domain scores, L2FC 1.05 FDR 0.02), even when corrected for mucosal inflammation (L2FC 1.22 FDR 0.002). *Lachnoclostridium* is a genus of particular interest in this regard. Despite not significantly associating with traditional disease severity indices, it was also observed in greater abundance in those with lower haemoglobin levels at diagnosis (L2FC -0.54 FDR 0.11), a change that is still present when corrected for mucosal inflammation with the composite FCP (L2FC -0.87 FDR 0.07).

5.3.1.5 Identifying the need for advanced therapies across IBD and predicting the early failure of conventional therapies in UC

In the post PROFILE era (Noor et al., 2024), those with moderate to severe CD should progress directly to an AT to obtain better treatment outcomes. That has been our practice at UHB throughout this study period. As such, microbial differences will follow clinical severity at presentation. In UC however, the picture is less clear-cut. Some with severe disease will respond promptly to conventional therapy with induction corticosteroids and mesalazine, whilst others will derive no benefit and need early and expedited treatment escalation. Microbial predictors of this outcome are therefore important both for identifying targets for microbiome manipulation and allowing earlier risk stratification. There are several changes in the 16S data set that are associated with an increased likelihood of requiring AT. Overall, a significant reduction in alpha diversity can be seen across IBD, with those going on to require AT tending towards a lower alpha diversity at presentation ***(Figure 5-7).***

When IBD was considered as a whole, multiple genus level differences were seen in those who went on to require AT, with enrichment of *Fusobacterium* (L2FC 2.2 FDR 0.16) and depletion of 16 SCFA producing *Clostridia* including *Subdoligranulum* (L2FC -1.8 FDR 0.10), *Fusicatenibacter* (L2FC -1.51 FDR 0.10) and *Agathobacter* (L2FC -1.80 FDR 0.11). For CD in isolation, there was no differential abundance at genus level that reached statistical significance. However, when looking at those with UC in whom steroid induction and mesalazine failed to obtain a durable response, a clear pattern emerged. There were 10 patients to whom this situation applied, 9 of whom progressed to an AT, and one managed with an immunomodulator (azathioprine) at the time of analysis. The pattern for enrichment of *Fusobacterium,*

Haemophilus and *Veillonella* was again observed, alongside significant increases *in Escherichia.Shigella* and *Aggregatibacter* ***(Figure 5-8)***.

Patients were also stratified by treatment response at 6 and 12 months, though this approach is susceptible to confounding from the influence of different clinician prescribing behaviours, and the receipt of less timely escalation across hospital sites. In these analyses across all IBD, only a single difference was seen at 12 months (increased abundance of *Blautia* [L2FC 1.70 FDR 0.10] in those who respond to treatment vs those who do not). No significant differences were seen at 6 months, or in any analyses split by subtype. As a surrogate marker of this I also undertook comparisons of those going on to require more than one AT, in comparison to those who have just needed one. This remains susceptible to delayed treatment switches in those without response and those who switch for reasons other than non-response, such as an infusion reaction. Across IBD, those requiring more than one AT demonstrated depletion of *Roseburia* at baseline (L2FC -4.33 FDR 0.10). There were no significant differences amongst IBD subtypes. No UC patients required surgery within the study period, though three patients with CD. Amongst these patients, a significantly higher abundance of *Ruminococcus Gnavus* (L2FC 5.23 FDR 0.02) was noted.

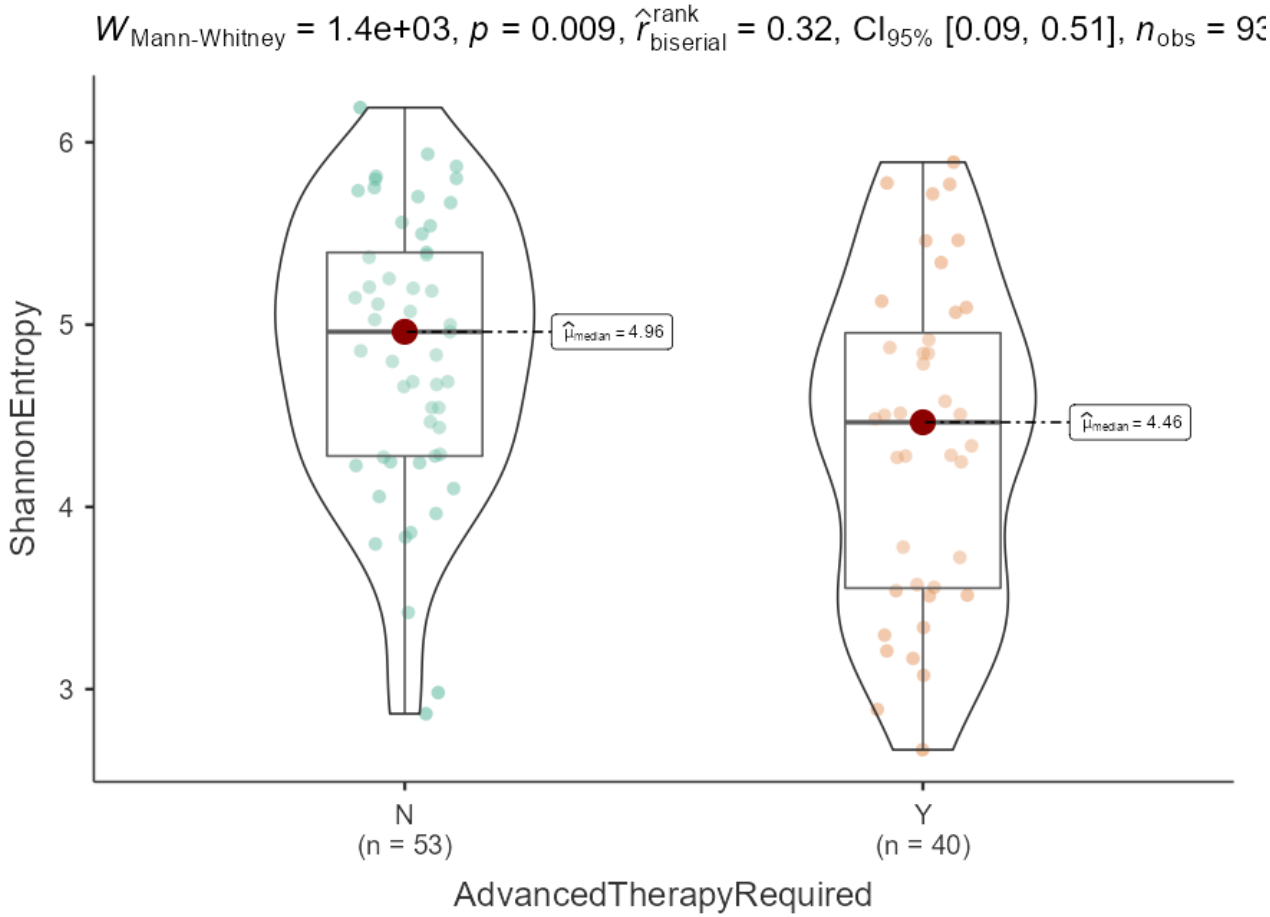


Figure 5 - 7: Baseline faecal alpha diversity in patients with IBD who go on to require AT, compared to those who do not.

Across IBD (n = 93, CD = 53 UC = 40), alpha diversity, as defined by the Shannon index, is seen to be significantly reduced in those who go on to require an AT (UC AT yes = 9, CD AT yes = 31). Alpha diversity in those requiring AT was a median of 4.46, compared to 4.96 in those who did not (Mann-Whitney 1400 p=0.009). These differences are just outside of statistical significance when the individual patient cohorts are analysed separately (CD yes = 4.48 vs no = 4.96 Mann-Whitney 435 p=0.09, UC yes = 4.27 vs no = 4.27 Mann-Whitney 199 p=0.056).

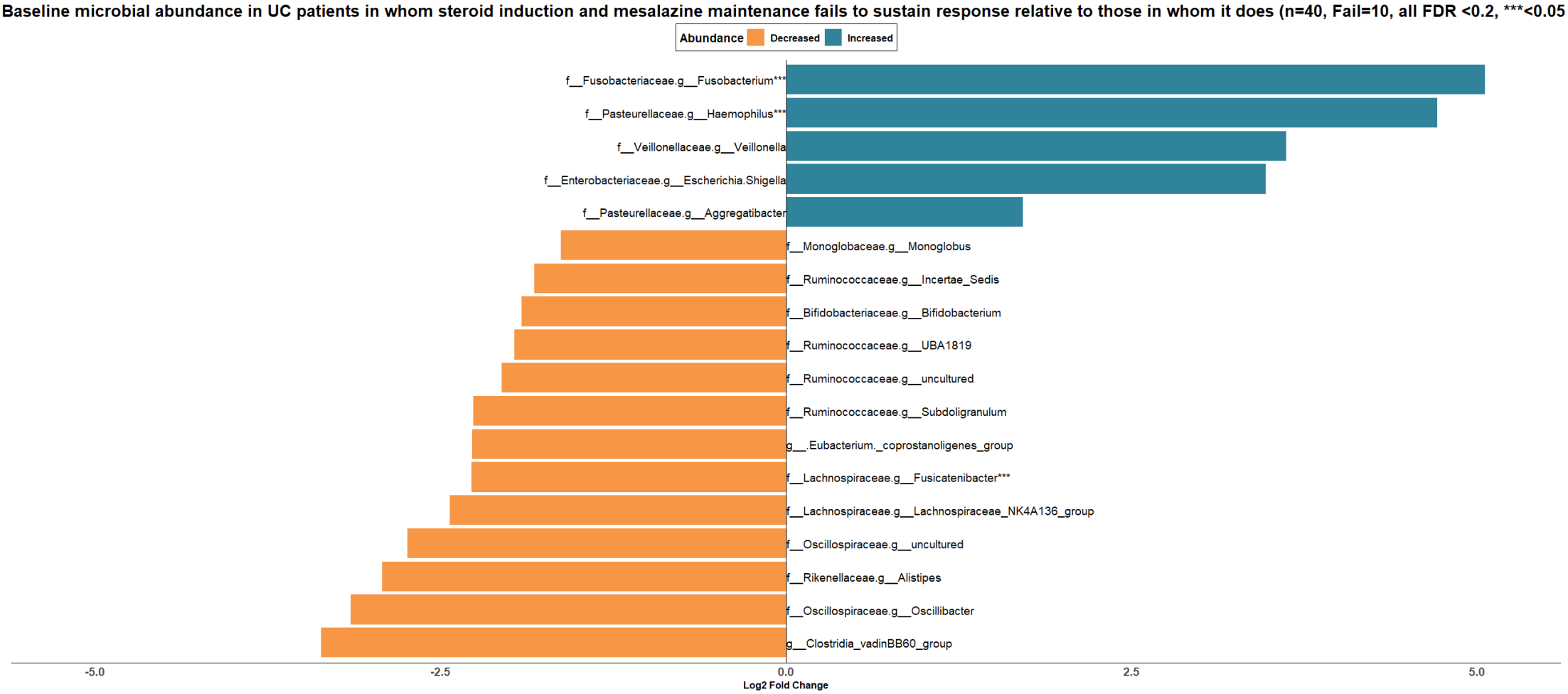


Figure 5 - 8: Baseline microbial abundance showing significant associations with the failure to maintain a response following steroid induction and mesalazine maintenance in Ulcerative Colitis.

Of 40 UC patients who did not have a durable treatment response to steroid induction and mesalazine (9 progressing to AT, 1 to azathioprine monotherapy) marked enrichment of typical oral pathobionts is seen; including *Fusobacterium* (L2FC 5.06 FDR 0.002) and *Haemophilus* (L2FC 4.7, FDR 0.01). Again, depletion is seen amongst typical short chain fatty acid producers including *Fusicatenibacter* (L2FC -2.28 FDR 0.03) and *Subdoligranulum* (L2FC -2.26 FDR 0.07).

5.3.1.6 Longitudinal comparison of pre and post treatment samples

Though attempts were made to draw out pre and post treatment differences, the available cohort was too small and too heterogenous in treatment response and disease state to draw out clear themes. Two patients have two separate post treatment samples, whilst a further 28 having one post treatment sample that has been sequenced thus far. No differences were observed below an FDR of <0.2.

5.3.2 Deeper characterisation of the IBD cohort using Shotgun Metagenomics

To further understand the changes seen in association with disease severity and behaviour, microbial DNA extracted from 85 stool samples was analysed using shotgun metagenomics. This included 65 patients already in the 16S dataset, with 20 additional patients included in whom DNA extraction was performed after the last 16S run or data quality from the 16S runs had precluded inclusion. In total, 48 patients with CD and 37 with UC were included. Full clinical indices were available for all of these, whilst disk data was collected for 68 (not collected in BHH) and galectin levels were available for 74. A median of 4.3 million reads were obtained per sample. This fell to 3.9 million after trimming and 3.6 million after decontamination. Once quality control steps had been undertaken, 7 patients were excluded from the analysis due to low microbial reads and high host content. Ultimately then, 78 patients were included, of whom 45 had CD 33 had UC.

5.3.2.1 Alpha Diversity

Initial analyses demonstrated no significant difference in alpha diversity between CD and UC patients on either Shannon (CD n=45 median 2.69, UC n=33 median 2.58 Mann Whitney U 832 p=0.368) or Simpson (CD n=45 median 0.86, UC n=33 0.87, p

0.282) metrics. Shannon diversity showed significant correlations endoscopic severity in CD (SESCD – r_s -0.353 p 0.016) and UC (UCEIS r_s -0.366 p 0.047). Significant correlations were also seen with the composite faecal calprotectin (n=76, r_s -0.33 p=0.004). In UC, no significant differences were seen in diversity when comparing between disease extent (as per Montreal classification). However, patients with ileocolonic disease had significantly lower alpha diversity at presentation than both those with isolated ileal or isolated colonic disease (n= 45 L1 n=18 median 2.88, L2 n=11 median 3.25, L3 n=16 median 2.07, L1vsL3 p=0.013, L2vsL3 p<0.001).

5.3.2.2 Beta Diversity

Beta diversity analyses were also undertaken in Vegan, using the Bray-Curtis index. Here, non-metric multi-dimensional scaling (NMDS) was utilised and visualised on a grouped plot (***Figure 5-9***). No major separation could be identified between UC and CD.

5.3.2.3 Species and strain level relationships with severity at IBD onset

There were no significant species level associations between either IBD subtype and FCP in the metagenomic dataset. Across grouped IBD, increases in faecal calprotectin were seen alongside a depletion of *Ruminococcaceae* including two strains of *Faecalibacterium Prausnitzii,* several different species of *Alistipes (Shahii, putredinis, communis, ihumii* and *finegoldii)* and *Lachnospiraceae* including *Coprococcus Eutactus,* and *Roseburia Intestinalis.* This is shown in ***Figure 5-10.***

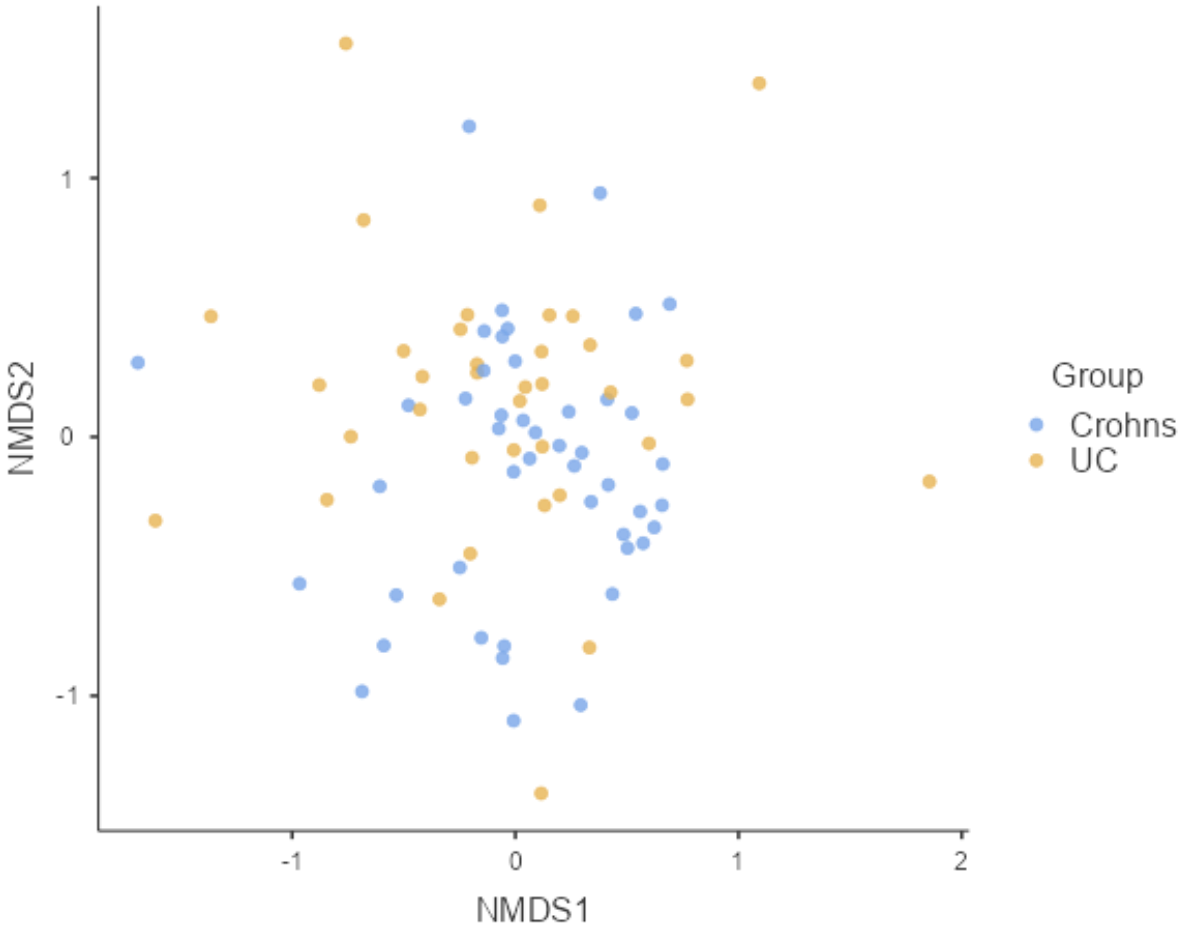


Figure 5 - 9: Beta Diversity, as determined using Bray-Curtis index and subsequent NMDS

This group plot displays NMDS values derived from beta diversity data for 78 pretreatment IBD patients (CD = 45 UC = 33).

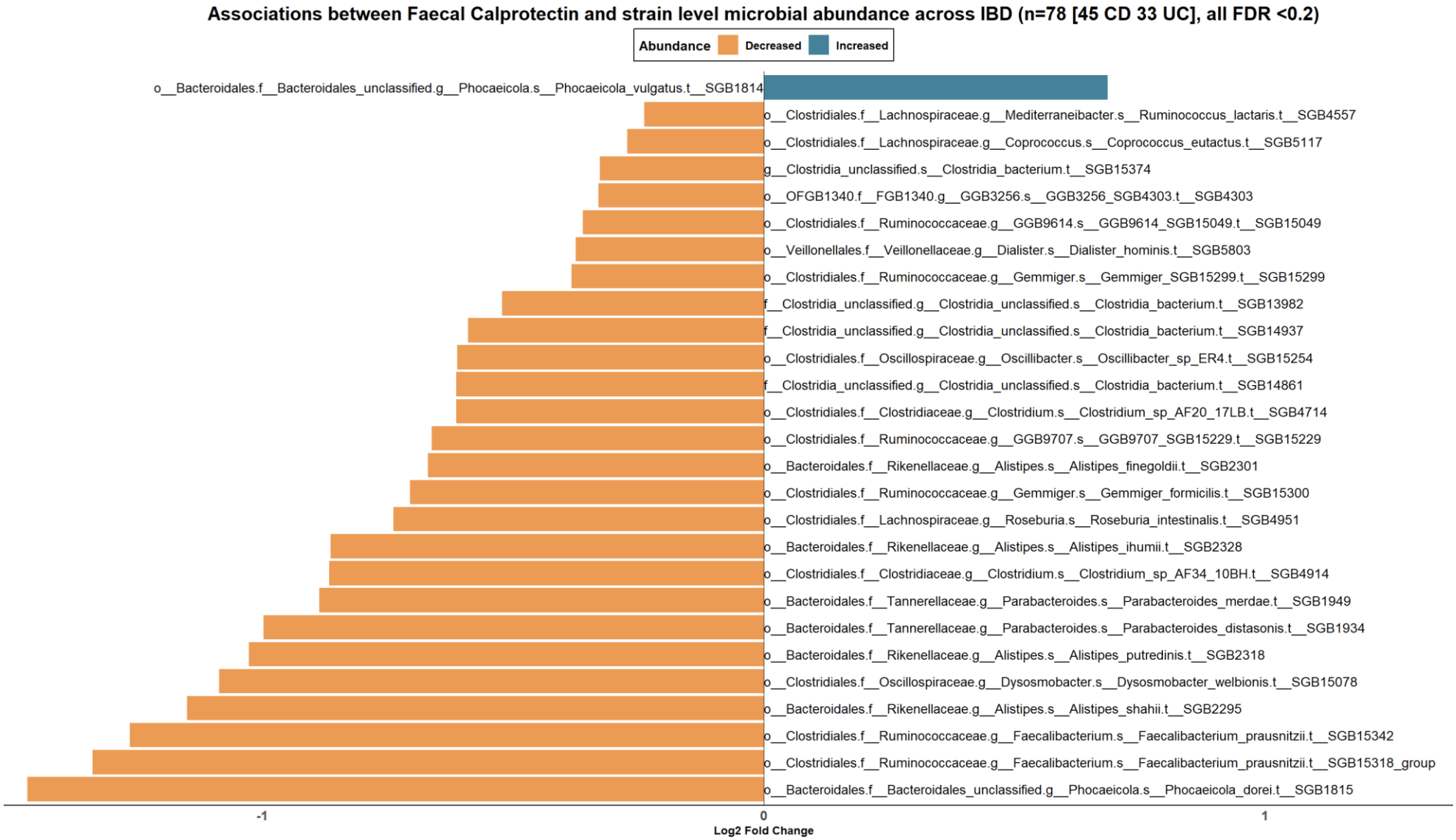


Figure 5 - 10: Strain level microbial abundance in relationship to increasing Faecal Calprotectin in IBD

As in the 16S data, the microbial abundance in the shotgun metagenomic dataset (n=78) has shown strong associations with faecal calprotectin, but on this occasion extending down to strain level in a mixed IBD cohort. 27 strains met significance thresholds with depletion of *multiple Clostridia* again seen to associate with higher FCP levels. *The* strongest associations were seen for depletion of *Faecalibacterium prausnitzii (SGB15318, L2FC -1.34 FDR 0.06; SGB 15342, L2FC -1.26 FDR 0.58)* and *Roseburia intestinalis* (SGB4951, L2FC -0.74 FDR 0.6)

In UC, similar microbial depletions were seen in relation to endoscopic severity (UCEIS). Depletion of *Faecalibacterium Prausnitzii (SGB15342,* L2FC -1.99 FDR 0.13) and *Ruminococcus lactaris SGB4557* (L2FC -0.79 FDR 0.12) were observed, alongside depletion of *Eubacterium rectale SGB4933* (L2FC -2.00 FDR 0.13) and *Bacteroides Uniformis SGB1836* (L2FC -1.61 FDR 0.13). In CD, depletion of *Faecalibacterium Prausnitzii (SGB15318)* correlated with increases in both endoscopic and clinical severity (HBI L2FC -1.91 FDR 0.03, SESCD L2FC -1.52 FDR 0.19). *Roseburia intestinalis (SBG4951)* associated with HBI (L2FC -0.98 FDR 0.01). Concordant signals were also sought regarding overall disability (IBD Disk) and psychological disturbance (*'Emotions'* domain), but none were identified.

5.3.2.4 Baseline associations with future outcomes

A key benefit of the metagenomic data was the ability to pursue signals regarding treatment outcomes from the 16S dataset beyond genus, down to strain level and whilst also assessing metabolic function. A reduction in baseline alpha diversity (Shannon) was observed in those going on to require AT, both in CD (n=45, AT yes n=32 median 2.34, AT no n=13 median 3.02 p=0.018) and UC (n=33, AT yes n=9 median= 1.57, AT no n=24 median 2.66 p=0.009). Across grouped IBD, enrichment of *Sutterella wadsworthensis SGB9286 was observed (L2FC 1.8 FDR 0.08)* alongside depletion of several familiar SCFA producing strains in those who subsequently went on to AT (***Figure 5-11***). When analyses were repeated in only CD, significant associations remain for depletion of *Fusicatenibacter saccharivorans SGB4874* (L2FC -2.2 FDR 0.15) and *Gemmiger formicilis SGB15300* (L2FC -1.78 FDR 0.15), with an additional signal for depletion of *Akkermansia muciniphila SGB9226* (L2FC -1.54 FDR 0.15).

As with the 16S data, for UC patients I sought to identify predictors of failing to obtain sustained remission via mesalazine therapy (with or without steroid induction). As before, this applied to 10 patients. Through this analysis, the prior genus level associations for *Fusicatenibacter, Oscillibacter* and *Alistipes* were followed down to strain level, with depletion of *Fusicatenibacter saccharivorans SGB4874* (L2FC -2.5 FDR 0.19), *Alistipes Onderdonkii SGB2303* (L2FC -384 FDR 0.19), *Alistipes Putredinis SGB2318* (L2FC -1.85 FDR 0.19) and *Oscillibacter sp ER4 SGB15254* (L2FC -2.3 FDR 0.19) identified.

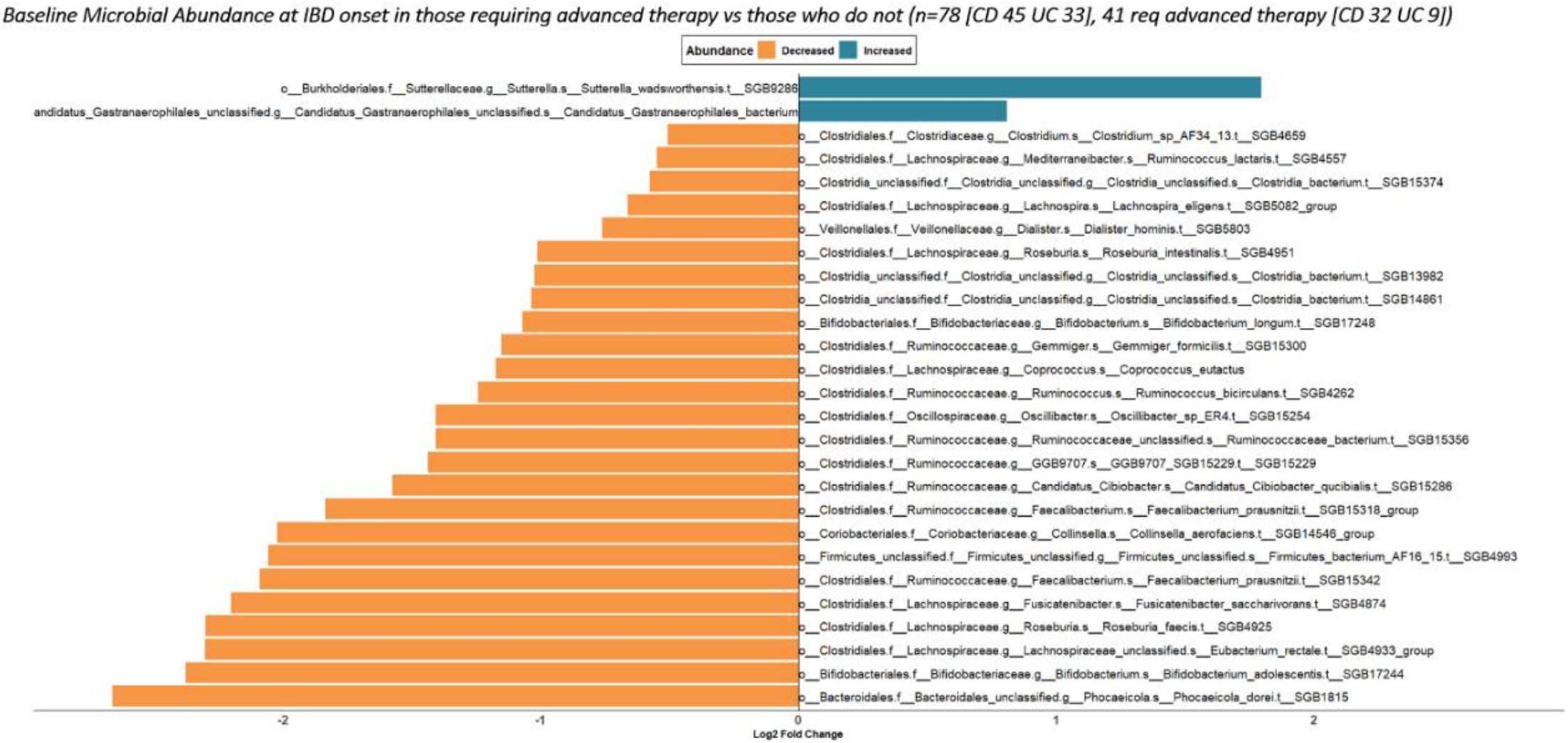


Figure 5 - 11: Strain level differential microbial abundance across 78 IBD patients at diagnosis, comparing those who went on to subsequently require AT with those that did not.

This plot compares 41 patients with IBD who went on to require AT (32 with Crohn's disease, 9 with Ulcerative colitis) to a further 37 patients who did not. Significant enrichment is observed *in Sutterella Wadsworthensis* SGB9286 (L2FC 1.8 FDR 0.08) with depletion of *multiple* Ruminococcaceae *and Lachnospiraceae* including *Fusicatenibacter saccharivorans SGB4874* (L2FC -2.2 FDR 0.04).

5.3.2.5 Differences in functional microbial metabolic activity at IBD onset

Whilst the abundance of specific strains of bacteria can provide insights into microbiome composition, it does not necessary correlate with the behaviour and metabolic activity of these bacteria. Through the metagenomic data it is possible to gain understanding of the metabolic activity of specific bacterial pathways and the functional contribution of specific species of bacteria to these.

In UC, increases in endoscopic severity (UCEIS) associated with reductions in metabolic activity from several species from the *Bacteroides* genus. This includes reduced activity of *Bacteroides Dorei* in 59 metabolic pathways, *Bacteroides Vulgatus* in 62 and *Bacteroides Uniformis* in 53 metabolic pathways. Reduced activity is also seen from *Eubacterium Rectale* across 34 pathways. The opposite signal is seen with regards to *Sutterella Wadsworthensis,* where small increases in metabolic activity are seen in 2 pathways. In CD, SESCD score is shown to associated with differential metabolic activity across 94 different pathways, albeit with differential activity only observed relating to one species, *Alistipes Putredinis,* which shows reduced activity in PWY.6609 (adenine and adenosine salvage III, L2FC -0.48 FDR 0.17).

Across IBD, differences in functional activity in association with overall disability (IBD disk) were not seen. However, if analyses factor in baseline FCP to adjust for inflammation, increased disability associates with increased activity of *Bacteroides caccae* and *thetaiotaomicron,* alongside reduced activity from *Eubacterium rectale* and *Faecalibacterium prausnitzii*.

Differential metabolic activity at baseline was sought amongst those who went on to require AT. When grouped together, minimal differences were seen, with reduced activity from *Eubacterium rectale* observed in one pathway. No significant differences were seen isolated to CD. For UC, again the failure mesalazine therapy / steroid induction was the desired metric. Here, I identified reduced metabolic activity across over 100 unique pathways. This included multiple pathways involved with amino acid synthesis including L-methionine, L-arginine and L-leucine, in addition to intermediates such as chromisate which is in turn involved in the synthesis of several essential metabolites *(**Figure 5-12**).* Pathways involved in SCFA production, such as P41.PWY and PWY.5100 pyruvate fermentation to acetate and lactate (I and II respectively) also showed reduced activity. Regarding specific microbial species, reduced activity relating to *Bacteroides vulgatus* was pronounced including in pathways central to SCFA synthesis (46 pathways including PWY66.429 fatty acid biosynthesis initiation mitochondria [L2FC -2.55 FDR 0.04] and COA.PWY1 superpathway of coenzyme A biosynthesis III mammals [L2FC 1.93 FDR 0.04]). Reduced activity was also observed for *Bacteroides dorei* (2 pathways) and *uniformis* (2 pathways); *Alistipes onderdonkii* and *finegoldii* (3 pathways respectively); and *Faecalibacterium prausnitzii* (1 pathway).

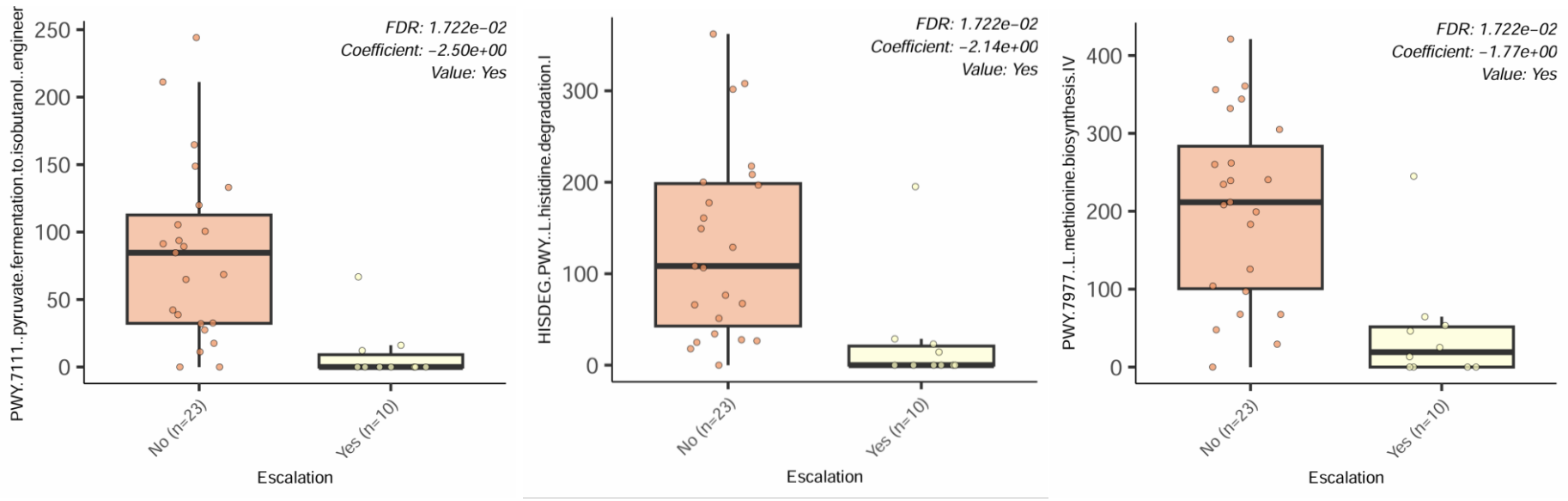


Figure 5 - 12: Three of the top 18 metabolic pathways that demonstrate reduced activity at baseline in patients with Ulcerative colitis who do not sustain responses to conventional mesalazine therapy and steroid induction, all with a false discovery rate adjusted q value of 0.017.

Across a dataset of 33 UC patients, of whom 10 did not respond to mesalazine and were escalated to an immunomodulator (1) or AT (9), significantly reduced metabolic activity was seen across multiple metabolic pathways. 18 of these carried a false discover rate (FDR) adjusted q value of 0.017, of which three are shown above. These are PWY.7111..pyruvate.fermentation.to.isobutanol..engineered. (L2FC -2.49 FDR 0.017), HISDEG.PWY..L.histidine.degradation.I (L2FC -2.14 FDR 0.017) and PWY.7977..L.methionine.biosynthesis.IV (L2FC -1.77 FDR 0.017).

5.3.3 An integrated analysis of the pre-treatment gut microbiome in IBD

Whilst our dataset, in the context of the literature, represents a large adult pre-treatment cohort, it is limited to faecal samples. Pooled re-analysis with publicly available sequence data, encompassing multiple sample types, age groups and geographic areas, is desirable to seek consistent differences. Clearly, to do this careful consideration must be given to methodology to account for the 16S rRNA hypervariable regions amplified for sequencing and the different read depths presented. As such, I have undertaken this endeavour with support from collaborators in the University of Sydney, University of Dundee and University of Central Lancashire.

5.3.3.1 Methods

All studies identified in the earlier systematic review and meta-analysis ***(Section 1.3)*** were screened for publicly available data derived from next generation sequencing. Where sequence data or usable clinical metadata were not available, requests were sent to individual authors to request data sharing. Given the scarcity of metagenomic data in this area, only data derived from amplicon sequencing was requested and amalgamated. Raw data and metadata were downloaded and amalgamated into one manifest file. Once pooled, these were re-run through a defined bioinformatic pipeline as one. Amplicon data was controlled again with DADA2 (Callahan, 2016) embedded in QIIME2 (Bolyen et al., 2019). Host contamination was removed using Bowtie2 (Langmead and Salzberg, 2012). Samples were only included in onward analysis where greater than 10000 clean reads were obtained. Taxonomy annotation was undertaken using a QIIME2 feature classified plugin with the Silva 138 database. R packages QIIME2R (Bisanz, 2018) and Phyloseq (McMurdie and

Holmes, 2013) were employed for diversity analyses. The Mann-Whitney test and Kruskal Wallis test were used for alpha diversity comparisons between groups. Adonis was used to assess the statistical significance of metadata variables between two distance matrices in beta diversity (Anderson, 2001). For differential abundance, Phyloseq and the R package Deseq2 were utilised (Love et al., 2014).

5.3.3.2 Results: Overview

As described in our earlier PRISMA, 61 full texts and 32 abstracts presented data from pre-treatment IBD patients. Ultimately, it was possible to obtain, either from public repositories or from direct data sharing, fastq files from 24 studies which had matched clinical metadata. An updated and shortened PRISMA is shown in ***Figure 5-13****,* to account for those studies excluded. After bioinformatic processing 6 further studies were lost; 5 of which related to data quality and 1 dataset was shared in a file format that was not usable.

As in ***Section 1.3,*** study summary characteristics are shown in a table ***(5-2)***, which also summarises the methodological differences and ROBINS-E risk of bias assessments. All studies entering the pipeline, including the 5 studies where data quality prevented further inclusion, are presented in the table with the proportion of samples that passed the quality control steps.

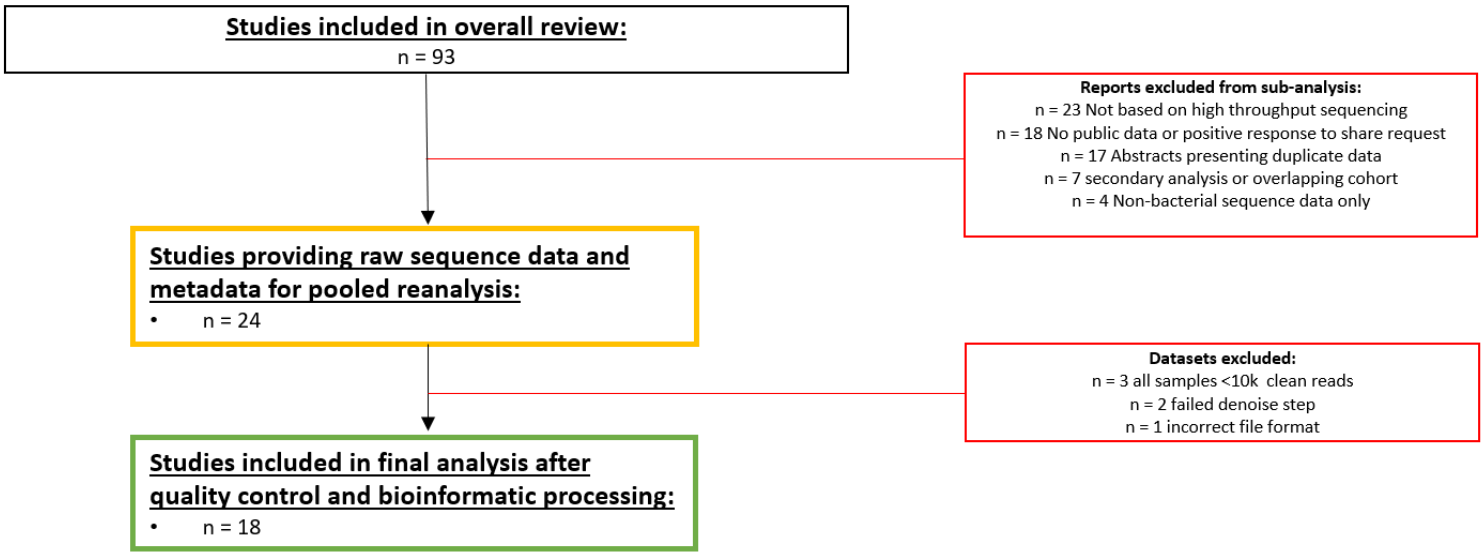


Figure 5 - 13: Study exclusions from secondary re-analysis
This abridged flow-chart represents a shortened PRISMA as presented earlier, showing the exclusion of 75 studies from our pooled secondary analysis of raw amplicon sequence data derived from pre-treatment IBD patients and controls

Table 5 - 2: Studies contributing raw amplicon data to the pooled re-analysis

AUTHOR	YEAR	SAMPLE TYPE	EXTRACTION KIT	PLATFORM	DOMAIN	DEPTH MEAN UNLESS STATED	COUNTRY	AGE GROUP	AGE (Overall in study) Mean (SD)	AGE (Overall in study) Median (IQR)	# PATIENTS WITH SAMPLES + METADATA	# SAMPLES PASSING BIOINFORMATIC QC n (%)	REASON FOR LOSS OF SAMPLE OUTPUT	RISK OF BIAS ROBINS E
Kaakoush et al.	2012	Faeces	Bioline ISOLATE fecal DNA kit	Roche 454	V1-3	2609	Australia	Paediatric	CD 11.6 (2.5) HC 9.5 (4.2)		CD 20 HC 22	0	All samples raw reads <10 000	Low
Hansen et al.	2012	Mucosal biopsy	Qiagen QIAamp mini kit	Roche 454	V3-4	21691	Scotland	Paediatric		CD 14.2 (ND) UC 13 (ND) SC 11.4 (ND)	CD 11 UC 11 SC 12	0	Failed in the denoise step	Low
Gevers et al. *	2014	Mucosal biopsy and faeces	Qiagen AllPrep mini kit	Illumina MiSeq	V4	29914	USA	Paediatric	CD 12.4 (3.0) SC 12 (3.7)		**Faeces** CD 166 SC 17 **Biopsy** CD 347 SC 217	851 (46.89)*	Metagenomic samples excluded	Low
Perez-Brocal et al.	2015	Mucosal biopsy and faeces	Qiagen QIAMP DNA stool mini kit	Roche 454	V1-3	10043	Spain	Adult	CD 45.4 (18.1) SC 33.5 (14.4)		**Faeces** CD 14 HC 24 **Biopsy** CD 14	2 (3.85)	Majority <10 000 clean reads	Some concern
Assa et al.	2016	Mucosal biopsy	FastDNA Spin Kit	Illumina HiSeq	V6	292215	Canada	Paediatric		CD 14 (4.75) SC 14 (2)	CD 11 SC 15	26 (100)		Low
Grover et al. * **	2016	Mucosal biopsy	ND	Illumina MiSeq	V6-8	ND	Australia	Paediatric	ND	ND	CD 23 SC 6	44 (51.76)*		Some concern
Mottawea et al.	2016	Mucosal luminal interface aspirate	FastDNA Spin Kit	Illumina HiSeq Roche 454	V6 V6	200000 13313	Canada	Paediatric		CD 14 (3.25) UC 15 (4.5) SC 14 (6)	**Illumina** CD 65 UC 23 SC 43 **Roche** CD 8 UC 8 SC 9	131 (83.44)	Mucosal-luminal aspirates excluded from analyses stratified by sample type as contribution only from one study	Low
Shah et al.	2016	Mucosal biopsy	Qiagen 'buffers'	Illumina MiSeq	V4-6	2350 rarefied	USA	Paediatric	UC 12.9 (3.7) SC 13.9 (1.8)		UC 9 SC 13	5 (22.73)	Other samples <10 000 reads	Low
Shaw et al.	2016	Faeces	Not stated	Illumina MiSeq	V4	66000 median	USA	Paediatric	ND	ND	CD 11 UC 3 HC 4 FC 6	23 (95.83)		High
Ashton et al.	2017	Faeces	MP biomedical faeces extraction kit	Illumina MiSeq	V4	ND	England	Paediatric	CD 13.6 (2.1) UC 10.1 IBDU 12.6 FC ND		CD 3 UC 1 IBD-U 1 FC 3	8 (100)		Some concern
Douglas et al. *	2018	Faeces	ND	Illumina MiSeq	V6-8	13815	Scotland	Paediatric	CD 12.7 (2.4) SC 12.8 (2.4)		CD 20 SC 20	20 (17.39)	Some <10 000 reads	Low

Schirmer et al. *	2018	Mucosal biopsy + faeces	**Bx**: Qiagen AllPrep mini kit **Faeces:** Chemagic DNA blood kit	Illumina MiSeq	V4	3000 rarefied	USA	Paediatric	UC 12.8 (3.3)		**Biopsy** UC 211 **Faeces** UC 264	395 (83.16)		Low
Xu et al.	2018	Mucosal biopsy	Qiagen QIAamp stool mini kit	Illumina HiSeq	V3-4	ND	China	Adult	UC 48 (14)		UC 10	2 (10)*	Majority <10 000 clean reads	Low
Kansal et al.	2019	Mucosal biopsy	Qiagen AllPrep mini kit	Illumina MiSeq	V2	9188	Australia	Paediatric		CD 12 (ND) SC 12.3 (ND)	CD 88 SC 66	0	File format incorrect	Low
Levine et al.	2019	Faeces	Mobio PowerFecal DNA kit	ND	V4-5	ND	Israel Canada	Paediatric	CD 14.1 (2.6)		CD 59	57 (96.61)		Low
Lloyd-Price et al. *	2019	Mucosal biopsy + faeces	Qiagen AllPrep mini kit	Illumina MiSeq	V4	10000 rarefied	USA	Mixed	CD 20.2 (11.3) UC 24.7 (15.3) SC ND		**Biopsy** CD 56 UC 23 SC 22 **Faeces** CD 14 UC 7 SC 3	130 (68.4)*		Low
Diederen et al.	2020	Faeces	FastDNA spin kit	Illumina MiSeq	V1-2	4237	Netherlands	Paediatric		CD 14 (3) HC 13 (5)	CD 27 HC 15	22 (52.38)		Low
Hart et al.	2020	Faeces	Mixed methods	Illumina MiSeq	V3	103341	Canada	Paediatric	CD 11.9 (3.2) UC 13.4 (2.0)		CD 19 UC 8	0	After filtering features with low abundance or observed in very few samples, zero remaining features	Low
Ellul et al. ***	2021	Faeces	Qiagen QIAamp stool mini kit	Illumina MiSeq	V1-2	11800	Malta	Adult	CD 37.8 (16.6) UC 47.4 (16.6) HC 44.7 (16)		'IBD' 56 HC 96	150 (98.68)	No metadata to identify IBD subtype. Excluded from subgroup analyses.	Low
Wang X et al.	2021	Faeces	EZNA soil DNA kit	Illumina MiSeq	V3-4	ND	China	Paediatric	'IBD' 10 (5.3) HC 7.1 (3.8)		'IBD' 80 SC 48 HC 27	142 (91.61)	No metadata to identify IBD subtype. Excluded from subgroup analyses.	Low
Wang Y et al.	2021	Faeces	Qiagen QIAamp stool mini kit	Illumina NovaSeq	V3-4	186189	China	Paediatric		CD 13 (3) HC 12 (2)	CD 23 HC 20	0	All samples have clean reads <10 000	Low
Galipeau et al.	2021	Faeces	Qiagen QIAamp stool mini kit	Illumina MiSeq	V4	12510	Canada	Unclear*****	UC 19.7 (7.2) FC 20.3 (7.3)		UC 7	7 (100)		Low
Paljetak et al.	2022	Mucosal biopsy	MasterPure DNA purification kit.	Illumina MiSeq	V3-4	7916 median	Croatia	Adult		CD 46 (ND) UC 31 (ND) SC 31 (ND)	**Biopsy** CD 10 UC 13 SC 26 **Faeces** CD 10 UC 12 SC 26	0	Failed in the denoise step	Low
Rimmer et al. **	2022	Faeces	Qiagen QIAamp stool mini kit	Illumina MiSeq	V4	45079	England	Adult	CD 37.3 (16.3) UC 40.5 (14.8) SC 38 (12.4)		CD 53 UC 41 SC 52	145 (99.32)		Low

Total samples included in final dataset (% from paediatric patients)	**All samples** **All IBD 1513 (87%)****** CD 881 (91%) UC 509 (86%) IBD-U 1 (100%) **All controls 647 (71%)** HC 130 (27%) SC 509 (82%) FC 8 (100%)	**Faeces** **All IBD 770 (79%)****** CD: 367 (85%) UC: 280 (83%) IBD-U: 1 (100%) **All controls 269 (45%)** HC: 130 (27%) SC: 131 (60%) FC: 8 (100%)	**Mucosal biopsies** **All IBD 655 (93%)** CD: 449 (95%) UC: 206 (89%) **All controls 335 (88%)** All control samples from symptomatic controls It was not possible to determine the age of patient for 39 SC	
# of variations in methods	**Location: 11** countries of origin **Sample type: 3**	**Extraction kit: 11** **Sequencing type: 1**	**Sequencing platform: 4** **Sequence domain (if amplicon): 10**	

* Some individuals provided >1 sample (e.g. faecal and mucosal biopsy samples or samples separated by biopsy location or inflammation status).

** Data published in abstract but been substantially expanded and shared by the authors. This data is unpublished but included in this review.

*** This publication was detected by the search in abstract. The raw data was retrieved from a subsequent full text publication outside of the search window (Rausch et al [2023]).

**** It was not possible to stratify beyond an overall diagnosis of IBD due to insufficient metadata for 122 (55% paediatric) patients who were excluded from subgroup analyses

***** Galipeau et al recruited adult and paediatric patients pre IBD diagnosis and longitudinally followed up to see if the disease developed. Whilst the study suggests that those recruited as children developed IBD as adults, the shared metadata did not include age and as such it does not appear in analyses stratified by age.

SD = Standard Deviation, IQR = Interquartile Range, ND = Not documented by authors

5.3.3.3 Results: The Impact of sample type and age group

Metadata from two studies did not allow stratification beyond an overall IBD diagnosis. One study included mucosal-luminal interface aspirates which were excluded from analyses stratified by sample type. The final presented analysis includes 2,160 samples (881 CD, 509 UC, 1 IBDU, 122 IBD 'not-specified', 130 HC, 509 SC, 8 familial control [FC]), originating from 1,743 unique individuals (678 CD, 399 UC, IBD-U 1, 122 IBD 'not-specified', 130 HC, 405 SC, 8 FC). 168 participants provided both biopsies and faeces, whilst 249 provided biopsies from >1 site. First, variation in microbial composition owing to differing baseline characteristics was quantified. Foremost, sample type was studied ***(Figure 5-14)***.There was no data from HC regarding mucosal microbiome . In the remaining patients, mucosal biopsies were characterised by enrichment of *Actinobacteria* (effect size [EF] 0.44 p_{adj}<.001), *Bacteroidota* (EF 0.32 p_{adj}<.001) and *Proteobacteria* (EF 0.26 p_{adj}<.001), alongside depletion of *Firmicutes* (EF 0.35 p_{adj}<.001) and *Fusobacteriota* (EF 0.21 p_{adj}<.001) compared to faecal samples. Alpha diversity was significantly reduced in mucosal biopsies (p_{adj}<.001) relative to faeces, with a significant difference in community structure also observed (R2 0.0388 p_{adj}<.001). Whilst this was observed across disease subtypes, community structure was most closely matched in UC.

As explored in the introduction, despite an emphasis on the development of the gut microbiome over the first 1000 days of life, published studies highlight ongoing differences between the paediatric and adult microbiome through childhood and into adolescence (Radjabzadeh et al., 2020, Agans et al., 2011, Hollister et al., 2015). Many of the studies within this dataset arose from paediatric cohorts and we opted to undertake both pooled and stratified analysis based on patient age (adults vs

paediatric studies). In analyses grouping all sample types and diagnoses, a significant reduction in alpha diversity was observed in children relative to adults (p_{adj}<.001). There was also significant separation in beta diversity (R2 0.0402 p_{adj}<.001).

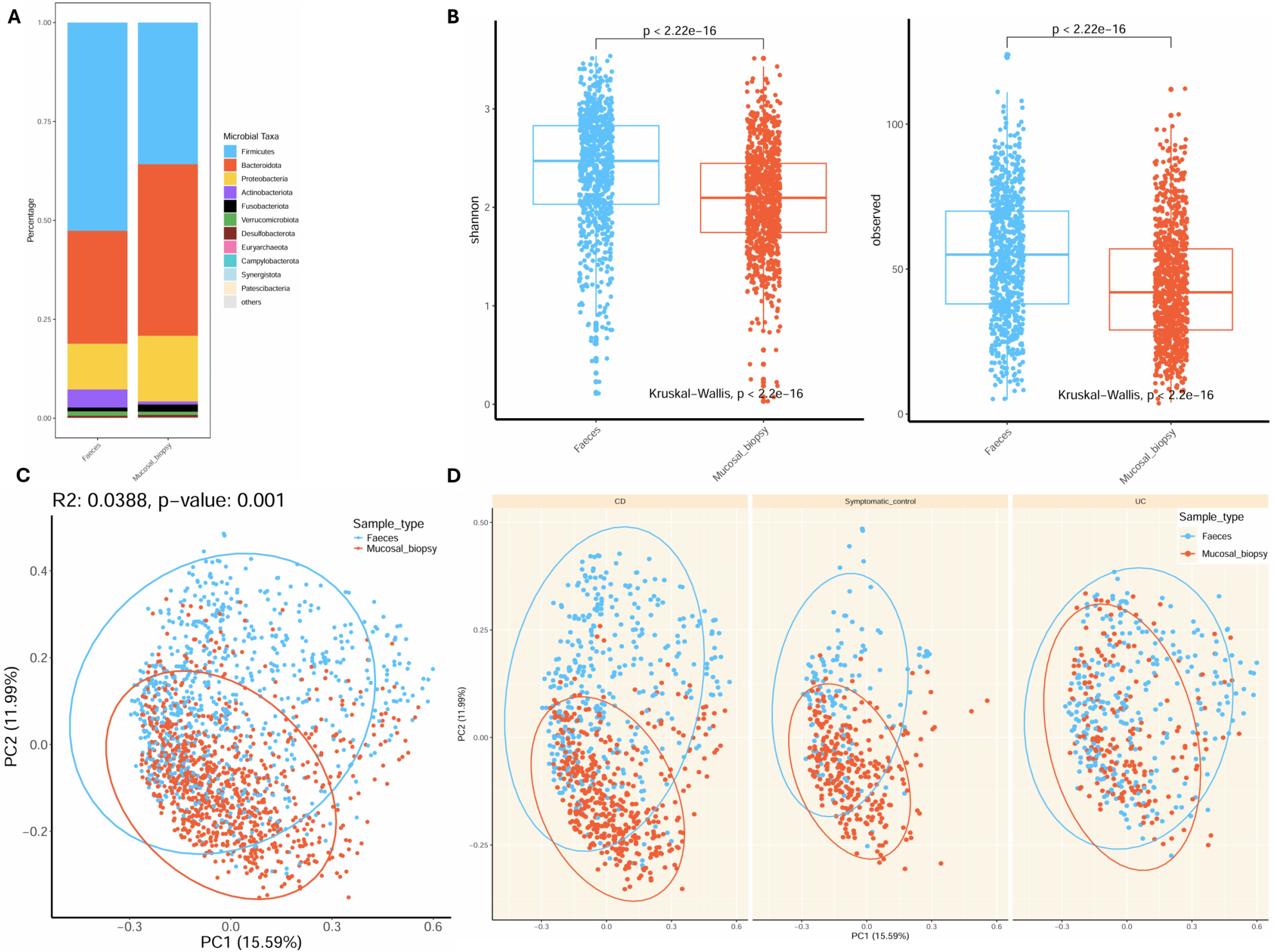

A
Percentage
1.00
0.75
0.50
0.25
0.00
Faeces
Mucosal_biopsy
Microbial Taxa
Firmicutes
Bacteroidota
Proteobacteria
Actinobacteriota
Fusobacteriota
Verrucomicrobiota
Desulfobacterota
Euryarchaeota
Campylobacterota
Synergistota
Patescibacteria
others
B
p < 2.22e−16
shannon
Kruskal−Wallis, p < 2.2e−16
Faeces
Mucosal_biopsy
p < 2.22e−16
observed
Kruskal−Wallis, p < 2.2e−16
Faeces
Mucosal_biopsy
C
R2: 0.0388, p−value: 0.001
Sample_type
Faeces
Mucosal_biopsy
PC2 (11.99%)
PC1 (15.59%)
D
CD
Symptomatic_control
UC
Sample_type
Faeces
Mucosal_biopsy
PC2 (11.99%)
PC1 (15.59%)

Figure 5 - 14: The difference in microbial community observed between mucosal biopsies and faecal samples in pre-treatment IBD and symptomatic control patients.

All panels presented subsequently exclude healthy controls since none of these control participants provided mucosal biopsies. Panels A - C are derived from 901 faeces samples (367 CD, 280 UC, 122 'IBD' patients [where metadata did not allow stratification] and 131 symptomatic controls) and 990 mucosal biopsies (449 CD, 206 UC, 335 symptomatic controls). Panel D includes only those where stratification is possible, so the number of faeces samples is reduced to 769 (367 CD, 280 UC, 131 symptomatic controls).

A: Microbial taxa bar plots at a phylum level. Faecal samples are characterised by a greater percentage of firmicutes (effect size [EF] 0.35 FDR adjusted $p<0.001$) and actinobacteria (EF 0.44 $p<0.001$), with a lower proportion of proteobacteria (EF 0.26 $p<0.001$) than mucosal biopsies.

B: Alpha diversity plots showing increased alpha diversity (Shannon index; faeces median 2.47, biopsy median 2.06 p [FDR adjusted] <0.001. Observed features; faeces median 53, biopsy median 41 $p<0.001$) in the faecal communities analysed as compared to the mucosal biopsies.

C: Overall Bray-Curtis beta diversity PCoA plot. Here we see clear separation between faeces samples and mucosal biopsies (R2 0.0388 $p<0.001$).

D: Bray-Curtis beta diversity PCOA plots split by diagnostic subtype. Significant separation is seen according to sample type across all diagnoses, though this difference is smallest in Ulcerative colitis (UC R2 0.0096 $p_{adj} = 0.015$, CD R2 0.0664 $p_{adj} = 0.015$, SC R2 0.0627 $p_{adj} = 0.015$).

5.3.3.4 Results: Revisiting alpha diversity in the pre-treatment IBD gastrointestinal microbiome.

Earlier (Section 1.3.2), I assessed alpha diversity (Shannon) indices presented by individual published studies and demonstrated significant reduced microbial richness/evenness in faecal samples, but not mucosal biopsies from both CD and UC. To challenge this conclusion, we undertook to repeat the analysis on the unified sequencing dataset. In paediatric faecal samples, significantly reduced Shannon alpha diversity was observed in UC relative to SC (p_{adj}=0.049) but not in CD. For observed features, reductions were significant in both UC (p_{adj}=0.02) and CD (p_{adj}<.001). Paediatric HC trended towards reduction in observed features relative to SC (p_{adj}=0.08). In adults, significant reductions were seen in alpha diversity in IBD relative to HC (CD p_{adj}<.001, UC p_{adj}<.001). Differences between IBD and SC were not significant. For controls, the opposite was seen to paediatric patients, with significantly increased Shannon diversity in HC relative to SC (p_{adj}<.001). This data is shown in ***Figure 5-15***.

For mucosal biopsies, most analyses were based upon paediatric samples, with no adult SC. Shannon alpha diversity was significantly reduced in paediatric CD compared to SC (p_{adj}<.001) and UC (p_{adj}<.001). Strikingly, Shannon alpha diversity in UC was significantly higher than SC (p_{adj}<.001). In the smaller adult cohort, no significant difference was observed between CD and UC. This data is presented in ***Figure 5-16***.

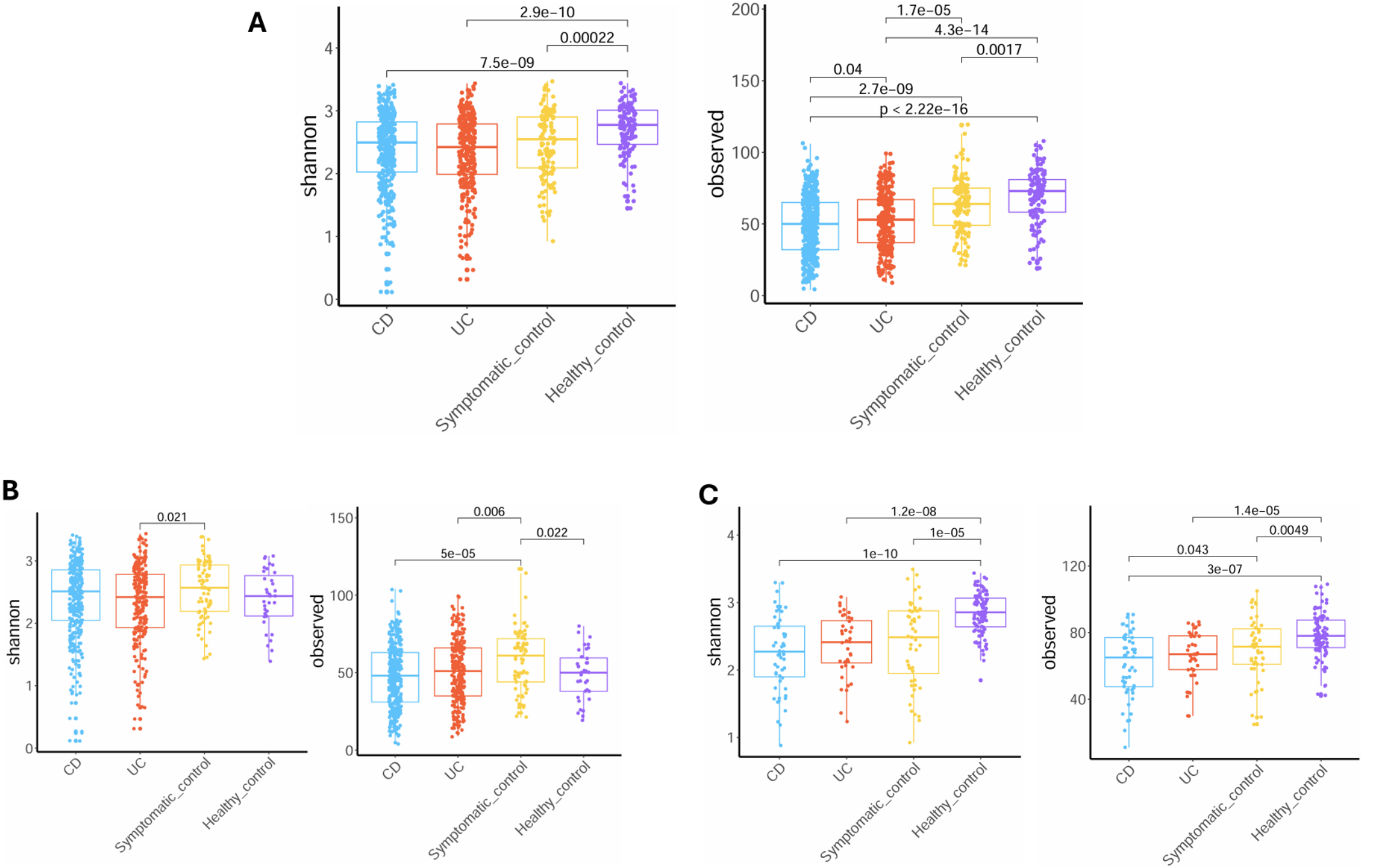

A
shannon
2.9e−10
0.00022
7.5e−09
observed
1.7e−05
4.3e−14
0.0017
0.04
2.7e−09
p < 2.22e−16
B
shannon
0.021
observed
0.006
0.022
5e−05
C
shannon
1.2e−08
1e−05
1e−10
observed
1.4e−05
0.0049
0.043
3e−07
CD
UC
Symptomatic_control
Healthy_control

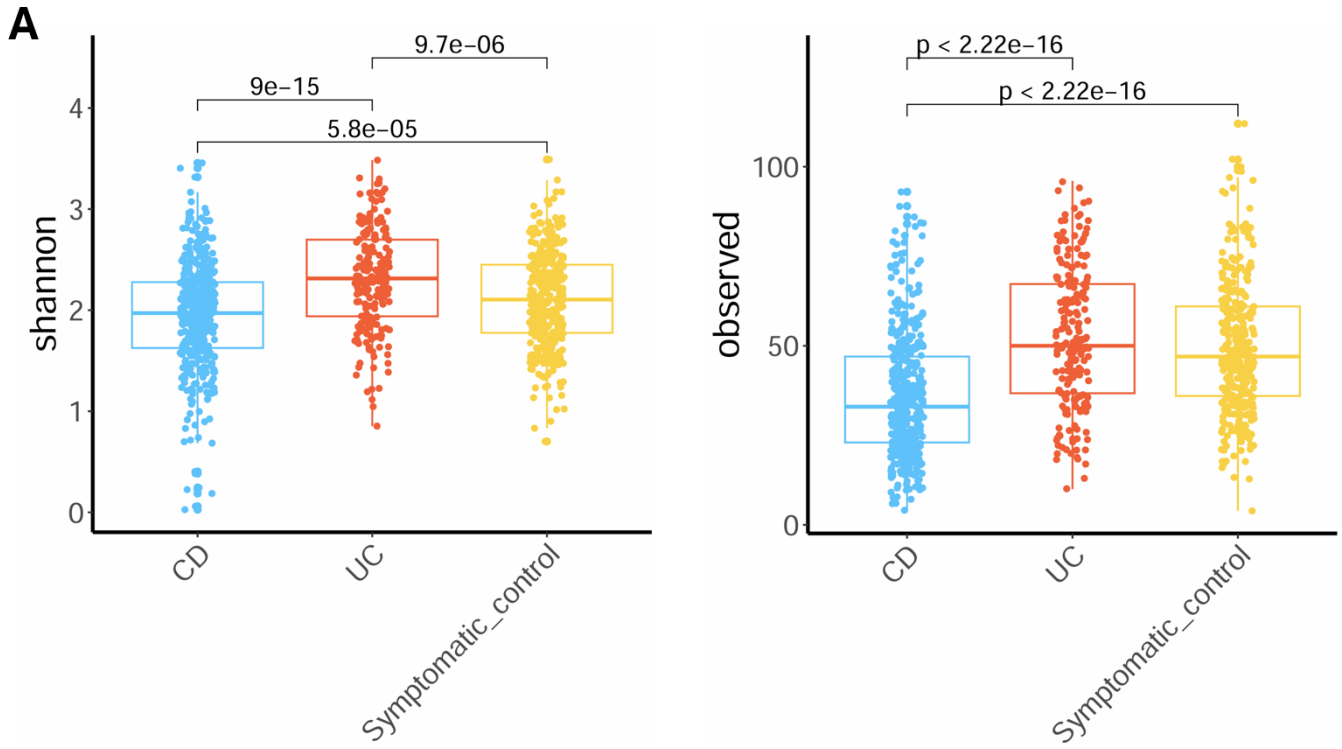
Figure 5 - 15: Faecal alpha diversity of pooled pre-treatment IBD patients and controls.

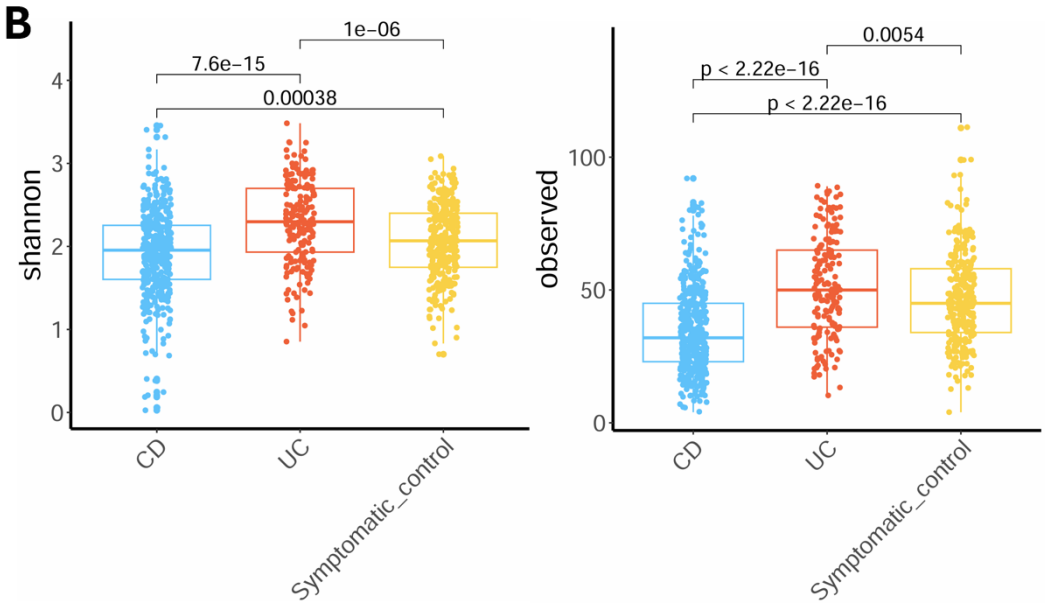
The P values on the plots above are not FDR corrected, though all p values within the figure legends are.

A: Alpha diversity plots are presented for both the Shannon index and observed features derived from faecal samples from both adult and paediatric patients (CD n=367, UC n=280, SC n=131, HC n=130). For the Shannon index, significant reductions in alpha diversity are seen in Crohn's when compared to healthy controls (CD median 2.50, HC median 2.78. Pairwise Wilcox test p<0.001). For UC, the difference with healthy controls is also significant (UC median 2.42, HC median 2.78. p<0.001). Non-significant differences are seen with symptomatic controls (SC median 2.55, CD p=0.28, UC p=0.098).

B: Again, alpha diversity is presented, however these plots only show data from paediatric patients (CD n=312, UC n=233, SC=79, HC=35). In this sub-analysis, the Shannon index is significantly reduced in UC as compared to symptomatic controls (UC median 2.42, SC median 2.57. p=0.049). Healthy controls in paediatric patients and adults have differing patterns of diversity, with a trend to reduction seen in children relative to symptomatic individuals. This does not reach significance for Shannon index (HC median 2.39, SC p=0.18). A stronger trend is seen in the plots of observed features, with significant reductions also observed between both CD and UC in comparison to symptomatic controls (CD median 48, UC median 51, SC median 61. UC p=0.02, CD p<.001).

C: Finally, alpha diversity plots are presented for adult patients only (CD n=76, UC n=62, SC n=52, HC n=95). We now observe symptomatic controls having significant reduced alpha diversity relative to healthy controls (Shannon index; SC median 2.48, HC median 2.85. p<0.001). For CD and UC patients, Shannon alpha diversity is reduced relative to healthy controls (CD median 2.27, UC median 2.39 HC median 2.85. p<0.001 for both). In the observed features plot, a significant reduction is also suggested in comparison of CD with symptomatic controls, though this does not stand after FDR correction (CD median 65, SC median 71.5. p=0.10).


A
shannon
9.7e−06
9e−15
5.8e−05
CD
UC
Symptomatic_control
observed
p < 2.22e−16
p < 2.22e−16


B
shannon
1e−06
7.6e−15
0.00038
CD
UC
Symptomatic_control
observed
0.0054
p < 2.22e−16
p < 2.22e−16

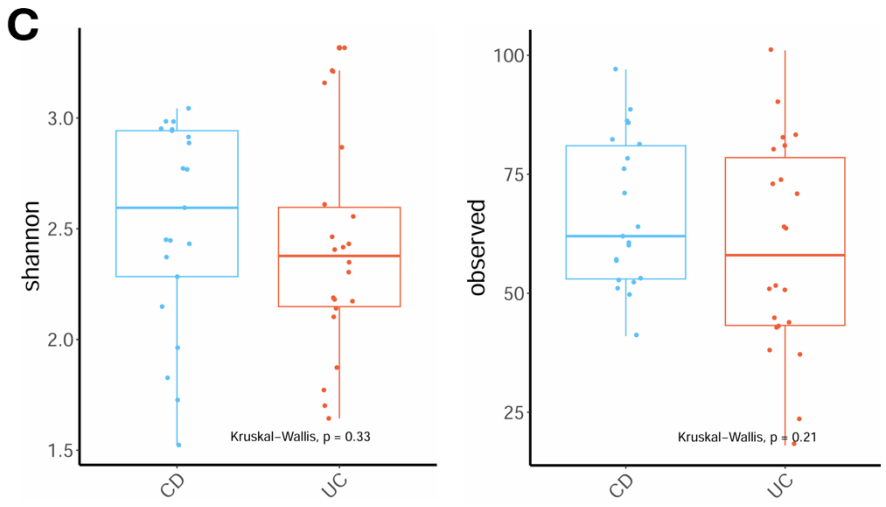

C
shannon
Kruskal−Wallis, p = 0.33
CD
UC
observed
Kruskal−Wallis, p = 0.21

Figure 5 - 16: Mucosal alpha diversity of pooled pre-treatment IBD patients and controls.

P values within the above plots are not FDR corrected, but all those within the figure legend are.

A: Alpha diversity plots are presented for both the Shannon index and observed features derived from gut mucosal biopsies samples from both adult and paediatric patients (CD n=449, UC n=206, SC n=335). Here we observed a clear trend for reduction in alpha diversity in patients with Crohn's disease. Regarding the Shannon index, this is the case for comparisons with both UC and symptomatic controls (CD median 1.97, UC median 2.31, SC median 2.11. Pairwise Wilcox test; CDvsUC $p<0.001$, CDvsSC $p<0.001$). Shannon index in UC is actually significantly higher than in symptomatic controls ($p<0.001$).

B: This panel again presents Alpha diversity, though it now presents it from only paediatric patients. This represents that majority of those providing mucosal biopsies (CD=428, UC=184, SC=296). Symptomatic controls are lost from Lloyd-Price et al (2019) as this is a mixed cohort and available metadata did not allow for the stratification of control patients by age group. The pattern of panel A is repeated with CD presenting significantly lower Shannon diversity than UC or SCs, with diversity in UC significantly higher than SCs (CD median 1.95, UC median 2.30 SC median 2.07, CDvsUC $p<0.001$, CDvsSC $p<0.001$, UCvsSC $p<0.001$).

C: Panel C presents Shannon and observed features in adult patients. It highlights first of all the paucity of mucosal biopsy data arising from adult patients IBD patients, with no defined controls present either. We also observed a reversal in the demonstrated pattern, albeit without statistical significance. Here, Shannon alpha diversity shows no significant difference between groups, though the overall value is higher in CD patients than UC (CD median 2.59, UC median 2.38 $p=0.47$).

5.3.3.5 Results: Microbial community structure

The integrated analysis was able to overcome our earlier inability to perform meaningful synthesis of community structure (beta diversity). Unified testing revealed clear separation between IBD, HC and SC populations in faecal samples. Significant differences with SC were also seen in the pooled adult and paediatric mucosal biopsy data ***(Figure 5-17)***. As with alpha diversity, differing patterns were also sought between adult and paediatric cohorts. Regarding paediatric faecal samples, all the overall pairwise comparisons remained significant upon sub analysis. For adults, all comparisons with HC remained significantly different. However, when comparing IBD subgroups to SC, no difference was seen with CD (R2 0.016 p_{adj}= 0.31) and differences with UC sat just outside of statistical significance after FDR correction (R2 0.027 p_{adj}=0.06).

For mucosal biopsy data, again no adult SC were included so comparative analyses were based only on paediatric data. Significant differences with SC were observed for both CD (R2 0.030 p_{adj}=0.003) and UC (R2 0.035 p_{adj}=0.003).

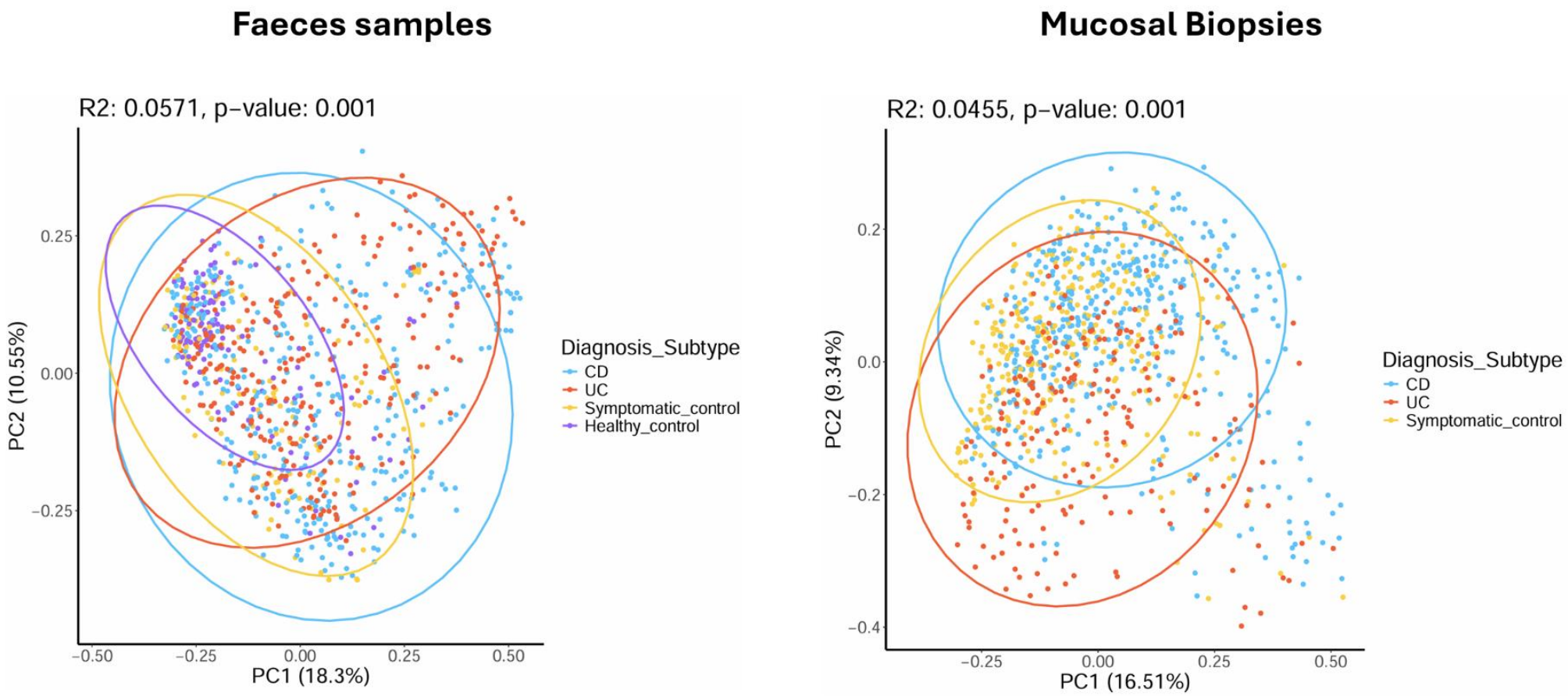


Figure 5 - 17: Beta diversity (Bray-Curtis) of faeces samples and mucosal biopsies from pooled adult and paediatric patients with IBD in a pre-treatment phase, in addition to control cohorts.

Overall analyses of faecal samples show significant differences between diagnostic subgroups across sample types. (R2 0.0571 p<.001). Upon pairwise comparisons, both CD (CD vs HC R2 0.053 p_{adj}=0.006, CD vs SC R2 0.020 p_{adj}=0.006) and UC (UC vs HC R2 0.068 p_{adj}=0.006, UCvsSC R2 0.039 p_{adj}=0.006) significantly separate from each control cohort. CD and UC also significantly differed from each other (R2 0.027 p_{adj}=0.006). For mucosal biopsies, overall analyses again demonstrate significant differences between diagnostic subgroups (R2 0.0455 p_{adj}<.001). For pairwise comparisons, both CD and UC significantly differed from symptomatic controls (CD vs SC R2 0.030 p_{adj}=0.003, UC vs SC 0.035 p_{adj}=0.003) and each other (R2 0.038 p_{adj}=0.003).

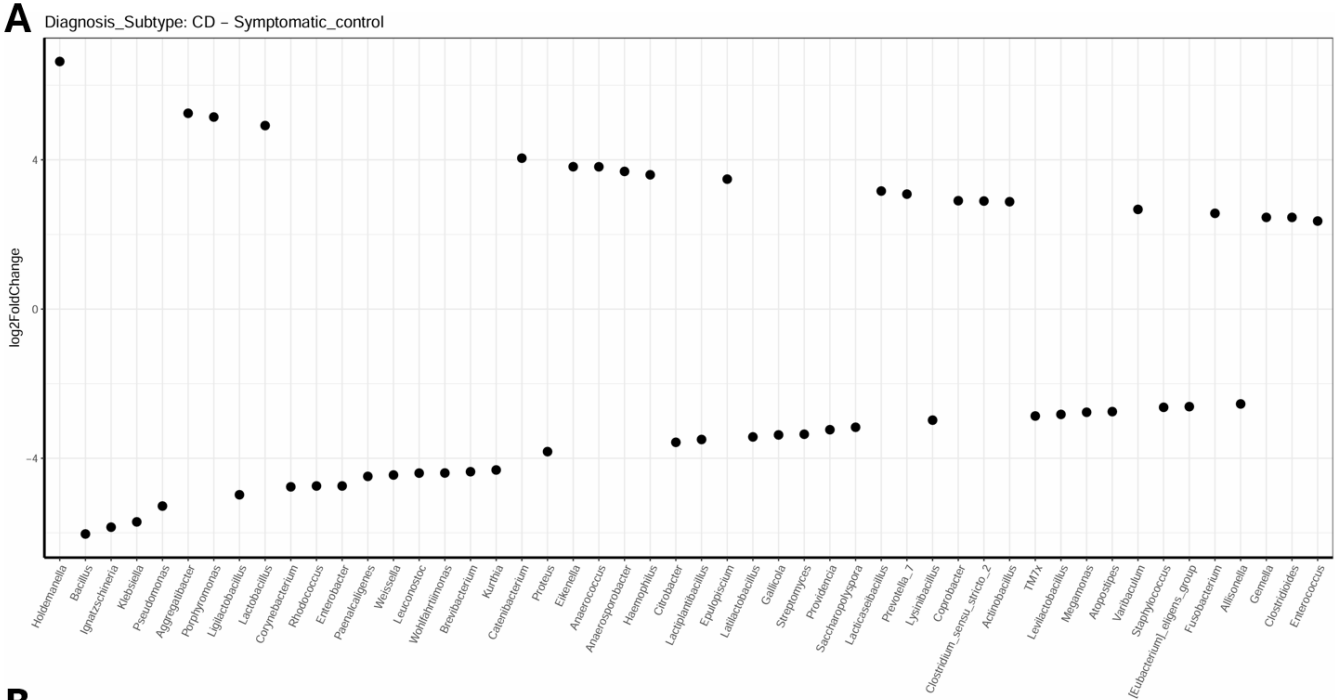
5.3.3.6 Results: Differential microbial abundance

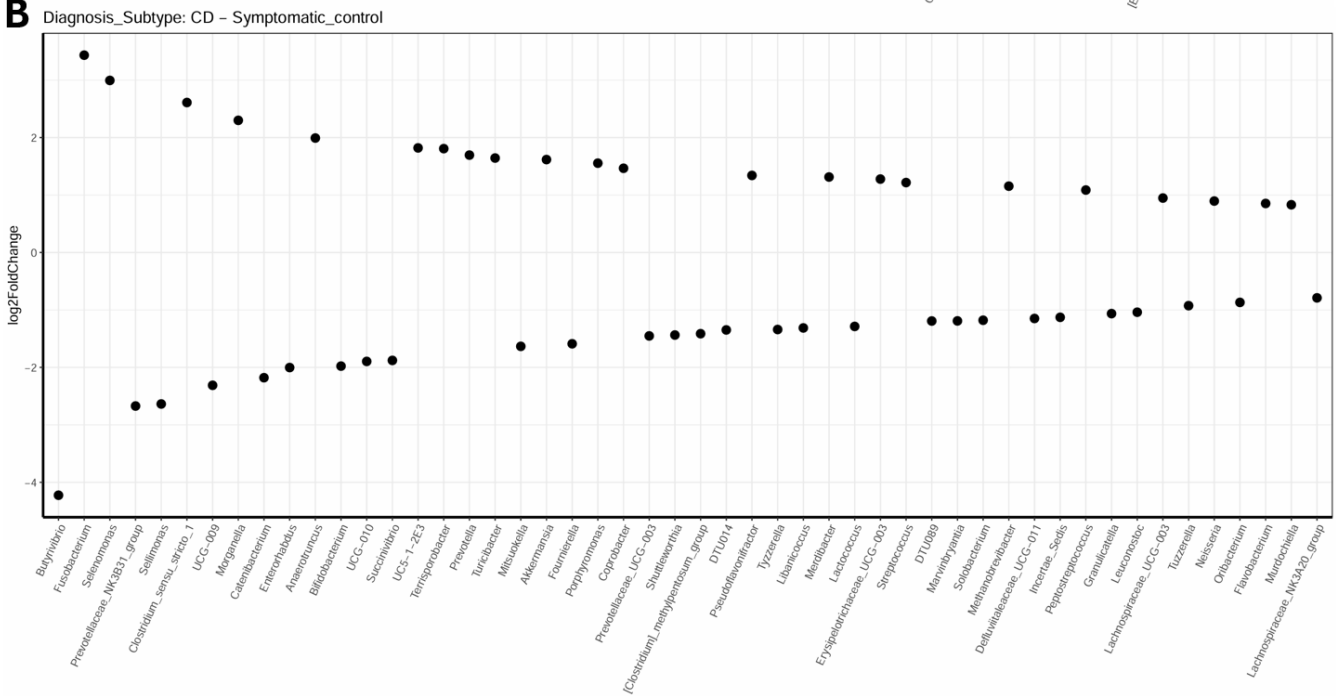
Differential abundance (genus) across sample types and diagnoses was interrogated using Phyloseq and DeSEQ2. SC were used as they were present in both faecal and biopsy datasets. Enrichment across genera typically found in the oral cavity was again conspicuous in both paediatric and adult samples.

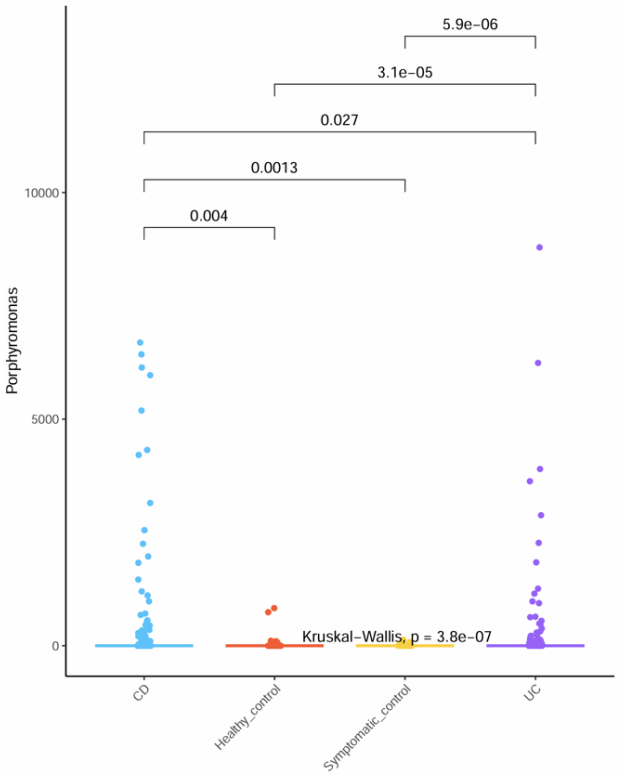
In CD, enrichment of *Aggregatibacter, Neisseria, Peptostreptococcus, Fusobacterium* and *Eikenella* was observed across all age groups and sample types. Depletion of known SCFA producers was also observed across ages and sample types including *Marvinbryantia, Terrisporobacter, Coprobacter* and *Anaerotruncus.* CD mucosal biopsies revealed additional signals for depletion of *Roseburia, Oscillibacter* and *Agathobacter.* These patterns are further explored in ***Figure 5-18*** and ***Figure 5-20***.

Similar patterns were observed in UC. Genera enriched across adult/paediatric datasets and sample types included *Haemophilus*, *Aggregatibacter*, *Peptostreptococcus*, *Porphyromonas*, *Neisseria* and *Gemella.* Depletion was also observed across these variables for genera associated with SCFA metabolism, including *Romboutsia, Clostridium Methylpentosum* and *Desulfovibrio.* Paediatric patients showed depletion of several *Eubacteria* (*Brachy, eligens, hallii, nodatum), Clostridium innocuum, Bifidobacterium, Oscillibacter and Phascolarctobacterium* in both biopsies and stool. This is highlighted in ***Figure 5-19*** and ***Figure 5-20***.

Crohn's Disease; Faecal samples, Deseq2







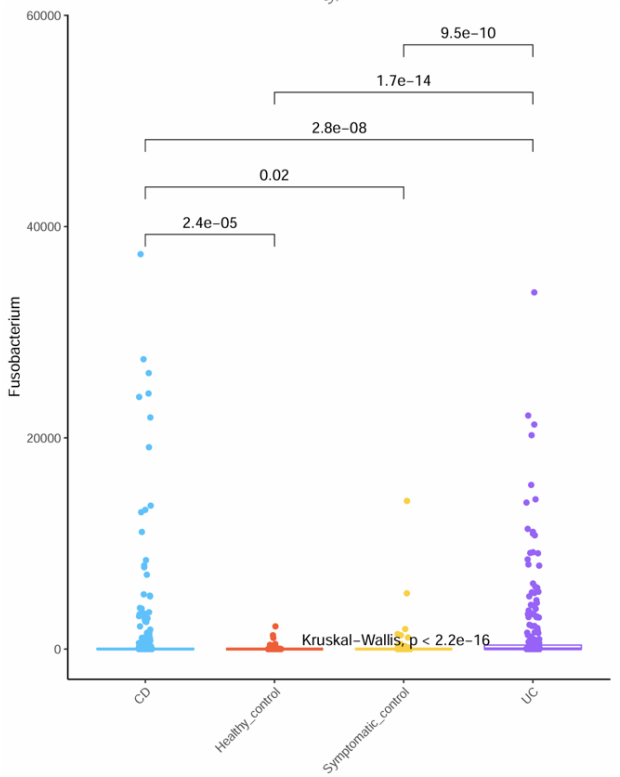


Figure 5 - 18: Differential microbial abundance observed in the faeces of patients with pre-treatment Crohn's disease, relative to symptomatic patients in whom IBD has been excluded.

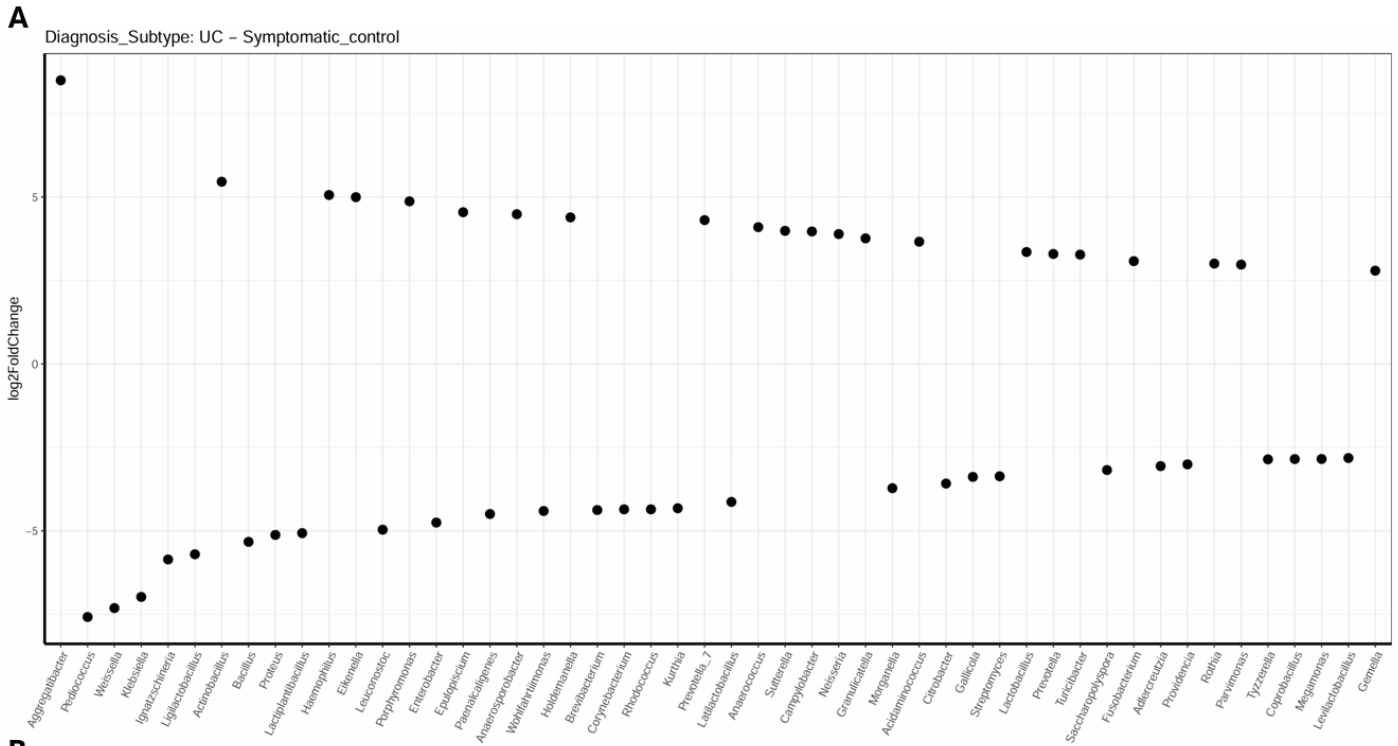
A: Panel A shows analysis, undertaken using deseq2, comparing microbial abundance at genus level in faecal samples obtained from paediatric patients with Crohn's disease (n= 312) and paediatric symptomatic controls (n= 79). The 3 genera (according to Log2 fold change) showing enrichment were *Holdemanella* (L2FC 6.64 p_{adj} <.0001), *Aggregatibacter* (L2FC 5.25 p_{adj} <.0001) and *Porphyromonas* (L2FC 5.15 p_{adj} <.0001). The 3 genera showing the greatest degree of depletion were *Bacillus* (L2FC -6.03 p_{adj} <.0001), *Ignatzschineria* (L2FC -5.84 p_{adj} <.0001) and *Klebsiella* (L2FC -5.7 p_{adj} <.0001).

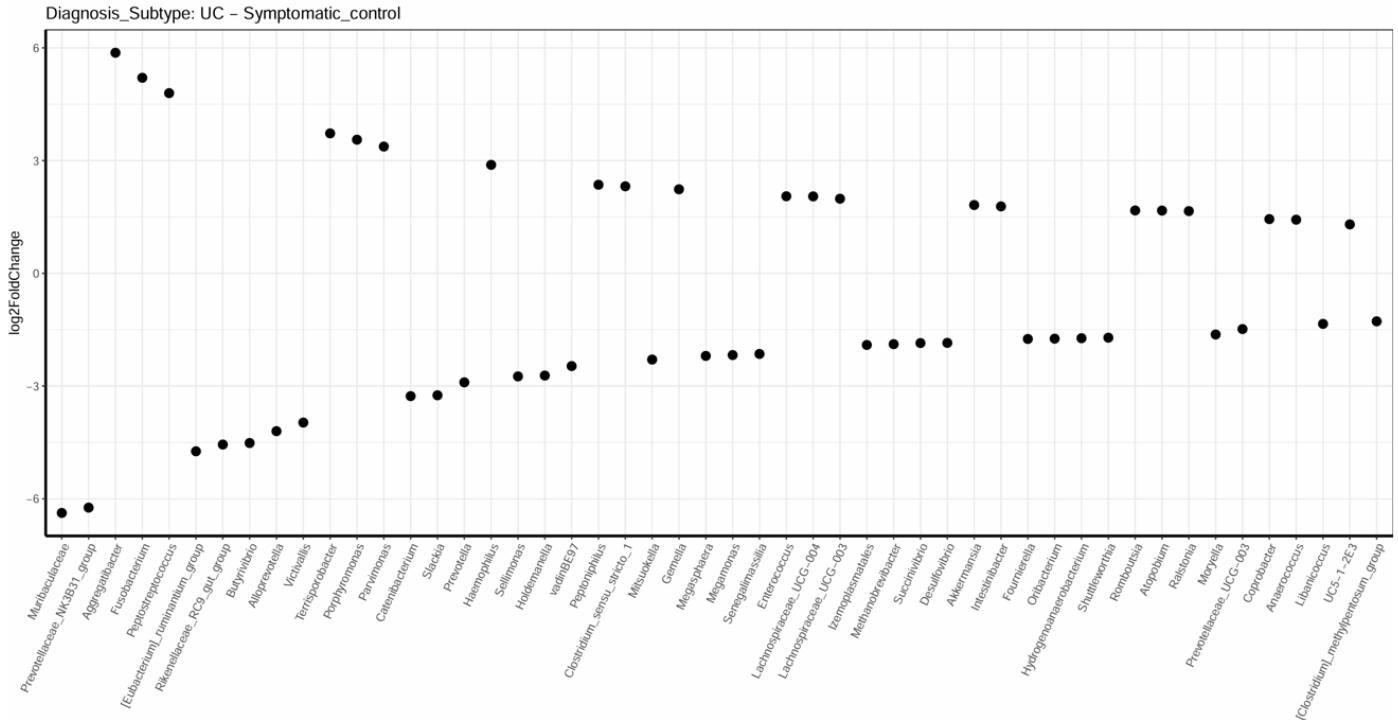
B: Panel B demonstrates further analysis from deseq2, now comparing adult patients with Crohn's disease (n= 55) and adult symptomatic controls (n= 52). The 3 genera showing enrichment were *Fusobacterium* (L2FC 3.43 p_{adj} <.0001), *Selenomonas* (L2FC 2.99 p_{adj} <.0001) and *Clostridium sensu stricto 1* (L2FC 2.61 p_{adj} <.0001). Enrichment concordant with the paediatric data was also observed in *Porphyromonas* (L2FC 1.55 p_{adj} <.0001). The 3 genera showing the greatest degree of depletion were *Butyrivibrio* (L2FC -4.22 p_{adj} <.0001), *Prevotellaceae NK3831 group* (L2FC -2.67 p_{adj} <.0001) and *Sellimonas L2FC -2.64* p_{adj} <.0001). Using deseq2 there were no concordant signals for microbial depletion. Indeed, *Catenibacterium* was enriched in children with CD relative to controls (L2FC 4.04 p_{adj} <.0001) but depleted in adults (-2.18 p_{adj} = 0.003).

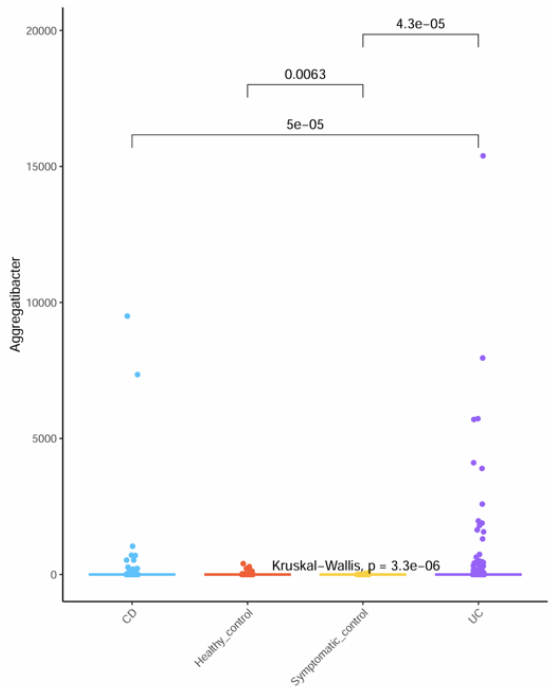
C: These plots demonstrate overall abundance of *Porphyromonas* and *Fusobacterium* across both the adult and paediatric dataset, to highlight the enrichment seen in the faeces of patients presenting with Crohn's disease (n = 367) relative to symptomatic controls (n= 141). P values presented on the plots are not FDR adjusted and are derived from a pairwise Wilcox test. The enrichment of *Porphyromonas* remains highly significant with FDR correction (p_{adj} = 0.005), though *Fusobacterium* falls outside of the most stringent thresholds for FDR significance (p_{adj} = 0.077).

Additional genera meeting statistical significance for enrichment or depletion across sample types but with low L2FC are not present on this plot, including *Neisseria* (Adult faeces L2FC 0.89 p_{adj} = 0.04, paediatric faeces L2FC 1.70 p_{adj} <.001*)* and *Peptostreptococcus* (Adult faeces L2FC 1.09 p_{adj} = 0.03, paediatric faeces L2FC 1.64 p_{adj} <.001).

UC; Faecal samples, Deseq2







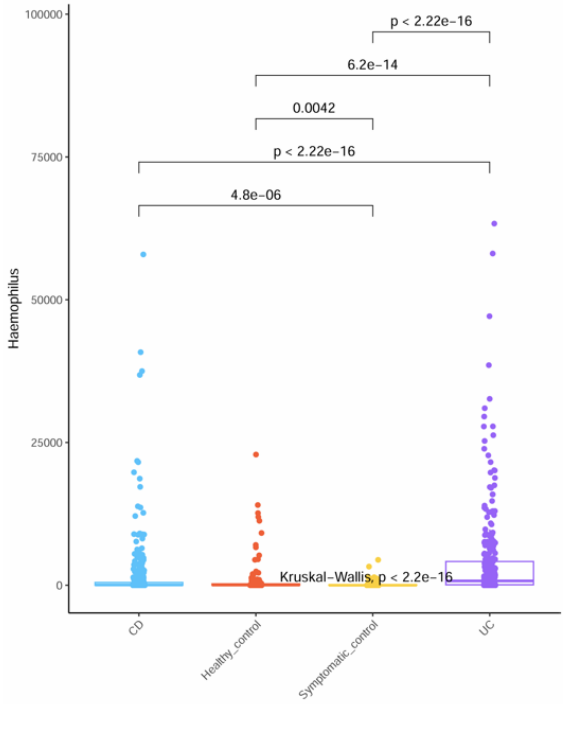


Figure 5 - 19: Differential microbial abundance observed in the faeces of patients with pre-treatment UC, relative to symptomatic patients in whom IBD has been excluded.

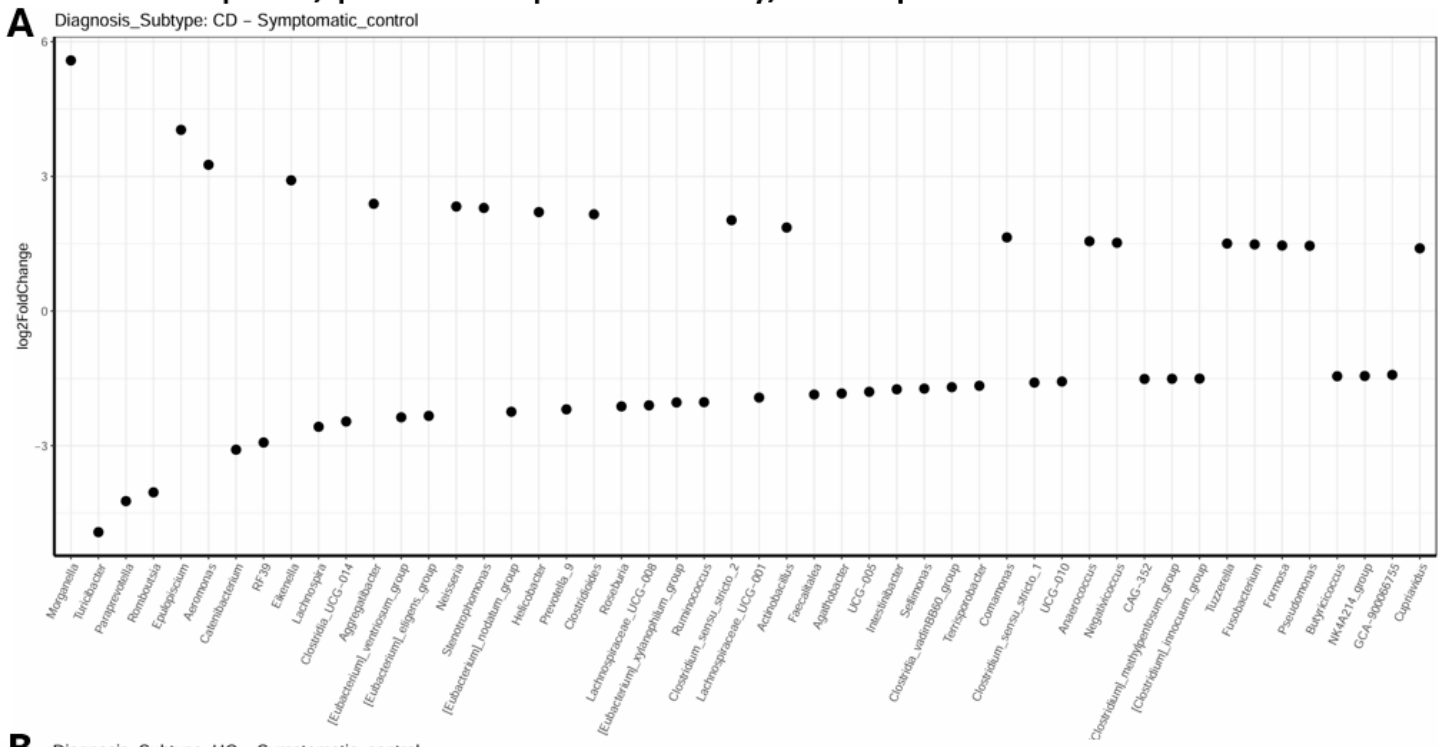
A: Panel A shows analysis, undertaken in deseq2, comparing microbial abundance at genus level in faecal samples obtained from paediatric patients with UC (n= 233) and paediatric symptomatic controls (n= 79). The 3 genera (according to Log2 fold change) showing greatest enrichment were *Aggregatibacter* (L2FC 8.49 p_{adj} <.0001), *Actinobacillus* (L2FC 5.46 p_{adj} <.0001) and *Haemophilus* (L2FC 5.06 p_{adj} <.0001). The 3 genera showing the greatest degree of depletion were *Pediococcus* (L2FC -7.58 p_{adj} <.0001), *Weissella* (L2FC -7.31 p_{adj} <.0001) and *Klebsiella* (L2FC -6.97 p_{adj} <.0001). The significant depletion of *Klebsiella* was also observed in paediatric CD.

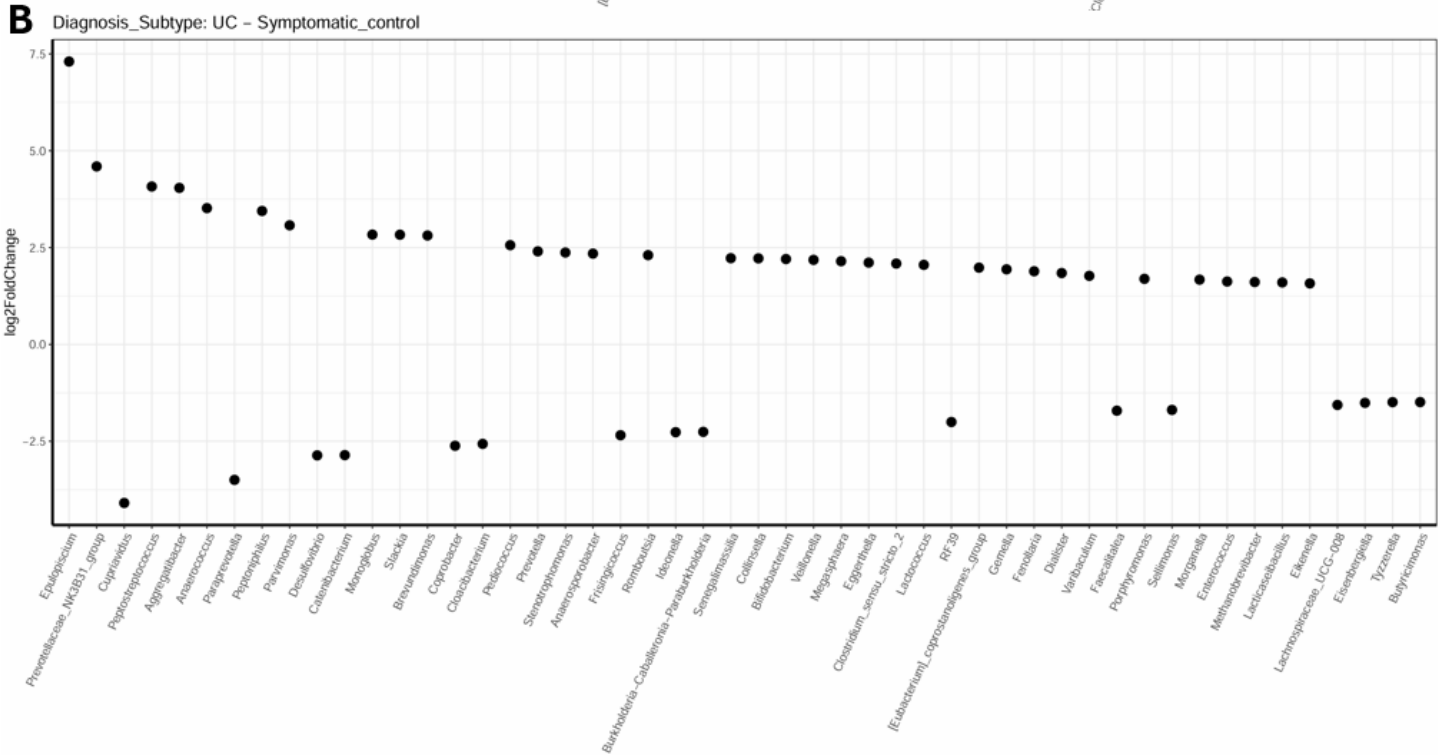
B: Panel B demonstrates further analysis from deseq2, now comparing faecal samples from adult patients with UC (n= 40) and adult symptomatic controls (n= 52). The 3 genera showing greatest enrichment were *Aggregatibacter* (L2FC 5.87 p_{adj} <.0001), *Fusobacterium* (L2FC 5.20 p_{adj} <.0001) and *Peptostreptococcus* (L2FC 4.79 p_{adj} <.0001). Enrichment of *Aggregatibacter, Fusobacterium, Haemophilus, Porphyromonas, Holdemanella and Gemella* was concordant across the adult and paediatric UC datasets. The 3 genera showing the greatest degree of depletion were *Muribaculaceae* (L2FC -6.38 p_{adj} <.0001), *Prevotellaceae NK3831 group* (L2FC -6.23 p_{adj} <.0001) and *Eubacterium ruminatium group* (L2FC -4.73 p_{adj} <.0001). Using deseq2, concordant signals for microbial depletion across adult and paediatric datasets were observed for again limited, with depletion of *Megamonas* observed across both.

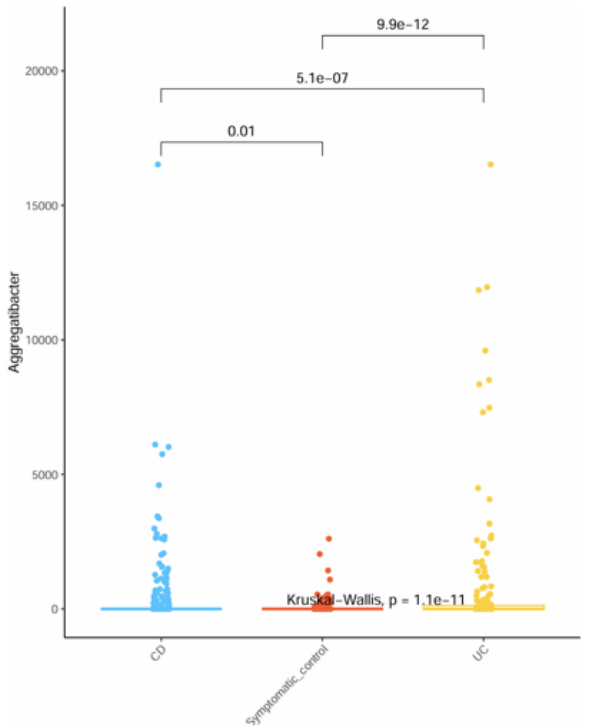
C: These plots demonstrate overall abundance of *Aggregatibacter* and *Haemophilus* across both the adult and paediatric dataset, to highlight the enrichment seen in the faeces of patients presenting with UC (n = 273) relative to symptomatic controls (n= 141). P values presented on the plots are not FDR adjusted and are derived from a pairwise Wilcox test. The enrichment of *Aggregatibacter* (p_{adj} <.0001) and *Haemophilus* (p_{adj} <.0001) remains highly significant with FDR correction.

Additional genera meeting statistical significance for enrichment or depletion across sample types but with lower L2FC are not present on this plot but can be found within the raw data and include paediatric enrichment of *Peptostreptococcus* (L2FC 2.20 p_{adj} <.0001) and adult enrichment of *Neisseria* (L2FC 1.18 p_{adj} = 0.007).

Mucosal biopsies; paediatric patients only, Deseq2







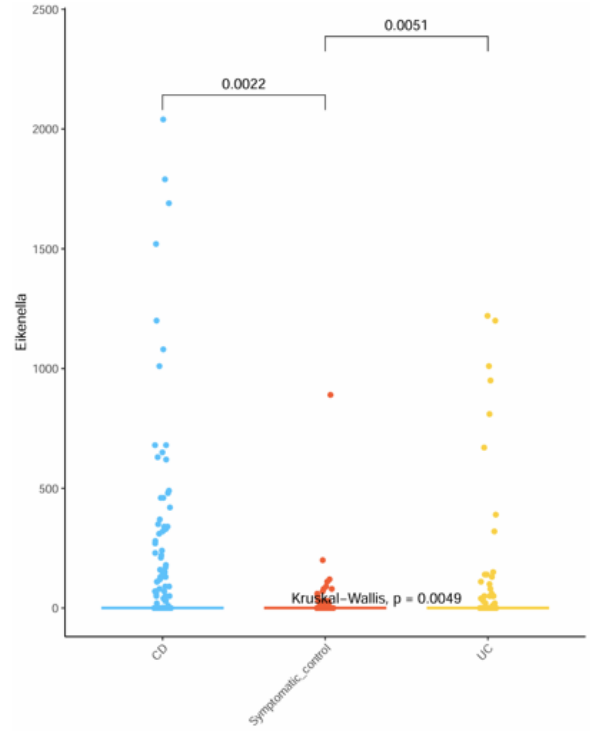


Figure 5 - 20: Differential microbial abundance observed in the mucosal biopsies of paediatric patients with CD and UC in comparison to biopsies obtained from symptomatic patients in whom IBD or other bowel inflammation has been excluded.

Adults are excluded from these plots entirely due to the absence of metadata confirming the presence of clearly defined adult symptomatic controls in the mucosal biopsy dataset.

A: Panel A shows analysis, undertaken in deseq2, comparing microbial abundance at genus level in mucosal biopsies obtained from paediatric patients with CD (n= 428) and paediatric symptomatic controls (n= 296). The 3 genera (according to Log2 fold change) showing greatest enrichment were *Morganella* (L2FC 5.58 p_{adj} <.0001), *Epulopiscium* (L2FC 4.04 p_{adj} <.0001) and *Aeromonas* (L2FC 3.26 p_{adj} <.0001). Enrichment of *Eikenella, Epulopiscium, Aggregatibacter, Fusobacterium, Peptostreptococcus* and *Neisseria* is observed in CD (relative to SCs) across both faecal samples and mucosal biopsies. The 3 genera showing the greatest degree of depletion in mucosal biopsies were *Turcibacter* (L2FC -4.92 p_{adj} <.0001), *Paraprevotella* (L2FC -4.23 p_{adj} <.0001) and *Romboutsia* (L2FC -4.04 p_{adj} <.0001). A number of genera also demonstrated discordant signals according to sample type. Examples include *Catenibacterium* (depleted in mucosal biopsies [L2FC -3.09 p_{adj} <.0001] and enriched in stool [L2FC 4.04 p_{adj} <.0001]) and *Pseudomonas* (enriched in mucosal biopsies [L2FC 1.45 p_{adj} <.0001] and depleted in stool [L2FC -5.28 p_{adj} <.0001]).

B: Panel B demonstrates further analysis from deseq2, now comparing mucosal biopsies obtained from paediatric patients with UC (n=184) and paediatric symptomatic controls (n=296). The 3 genera showing greatest enrichment were *Epulopiscium* (L2FC 7.30 p_{adj} <.0001), *Prevotellaceae NK3831 group* (L2FC 4.59 p_{adj} <.0001) and *Peptostreptococcus* (L2FC 4.08 p_{adj} <.0001). Enrichment of *Aggregatibacter, Haemophilus, Porphyromonas, Eikenella, Peptostreptococcus and Gemella* was observed across faeces and mucosal biopsies from paediatric UC patients. The 3 genera showing the greatest degree of depletion were *Cupriavidus* (L2FC -4.09 p_{adj} <.0001), *Paraprevotella* (L2FC -3.50 p_{adj} <.0001) and *Desulfovibrio* (L2FC -2.86 p_{adj} <.0001).

C: These plots demonstrate overall abundance of *Aggregatibacter* and *Eikenella* across mucosal biopsies from both CD and UC, in comparison to symptomatic controls. This is to highlight the enrichment of these genera across diagnostic subtypes, but also as key themes across different sample types. The p values displayed on the plots are not FDR corrected, but the statistical significance remains after this is undertaken after a pairwise Wilcox test. This applies to both *Aggregatibacter* (CD vs SC p_{adj} 0.04, UC vs SC p_{adj} <.0001) and *Eikenella* (CD vs SC p_{adj} 0.01, UC vs SC p_{adj} 0.02).

5.4 Discussion

The 16S dataset from the Birmingham cohort represents the largest adult IBD inception microbiome dataset in the literature. It provides additional insights regarding the differences in faecal microbiome composition between IBD subtypes at disease onset and SC. The sequence data is of a high quality, with 99.3% of our data passing the stringent quality control measures we applied for our meta-analysis.

Comprehensive analysis of microbial composition in the gastrointestinal tract is crucial for establishing associations between specific taxa and healthy and disease states. Data derived from amplicon sequencing of the 16S rRNA gene is currently the most widely available in published studies. Whilst metagenomics allows deeper analysis, a paucity of data in pre-treatment IBD meant that integrating and re-analysing our 16S data alongside published amplicon sequencing datasets offered the greatest potential to strengthen our conclusions. In having done this, we are now better placed than those before us to quantify the impact of methodology and sample type. Consequently, we can facilitate more robust interpretation of microbial sequence data in this space. Whilst others such as Gevers et al. (2014) cautioned against the use of faecal samples as a surrogate for mucosal biopsies in CD, we are now able to identify core themes that persist amongst different sample types and IBD subtypes. Furthermore, we can highlight key differences between adults and paediatric populations who present with IBD earlier in life.

Our meta-analysis of individual published studies ***(Section 1.3)*** yielded different findings compared to the unified bioinformatics approach. Appreciating that based on

data availability there were slight differences between analytical datasets, but noting the limitations of undertaking the traditional meta-analysis approach, the meta-analysis was unable to accurately represent the true differences in pooled microbiome datasets. This highlights the superiority of generating a unified bioinformatics approach with consistent QC standards. Impacts from sample type and age group can also be quantified. This study is the first of its kind in the microbiome field with an emphasis on pre-treatment IBD.

Performing an integrated analysis of raw data such as this will never be without limitation. Non-uniform bio-sample processing and inclusion of data derived from a multitude of 16S rRNA hypervariable regions introduces potential uncertainty. Disproportionately large contributions from some studies will also influence the conclusions. For example, Gevers et al. (2014), contributed 341 mucosal biopsies and 223 faecal samples from CD. This represents 76% of mucosal biopsies and 61% of faecal samples for this condition. A paucity of data from adults, particularly mucosal biopsies, limits these analyses and enriching their availability should be a priority for the research community. An additional limitation was access to datasets. Despite a move towards data availability and transparency, this was not always available, and high-quality metadata proved unobtainable in some cases, with some studies not even stratifying their results by IBD type. Metadata for disease severity and treatment outcomes was inconsistently reported. No single outcome was used and described regularly enough to me to repeat the analyses for disease severity presented when analysing my own data in isolation. Whilst my own dataset included metagenomics, the integrated dataset includes only amplicon sequencing. Accordingly, microbial abundance couldn’t reliably be determined beyond genus

level and direct data regarding microbial function was not available. Finally, meaningful investigation of non-bacterial microbial domains was not possible due to a dearth of available pre-treatment data.

5.4.1 The significance of microbial diversity at IBD onset

In our pooled paediatric data, faecal alpha diversity is reduced in UC compared to SC but not HC, with no significant reductions in CD. HC alpha diversity was lower than SC. The opposite is seen in adults, where faecal alpha diversity is increased in HC over SC. Furthermore, in adults, reductions in faecal alpha diversity are observed in comparisons of both UC and CD with HC but in neither when compared to SC. In mucosal biopsies, alpha diversity is reduced in paediatric CD but increased in UC compared to SC. These divergent patterns are likely multifactorial. First, CD mucosal biopsies can be obtained directly from the site of inflammation in CD rather than using a faecal sample as a surrogate for distant ileal inflammation. In UC, mucosal biopsies may be less impacted than the faeces by dietary and environmental factors including faecal transit time (Procházková et al., 2022). The mucosal microbiota may also have a closer relationship to the host phylogeny, though this remains debated across animal and human studies (Muegge et al., 2011, Gomez et al., 2019, Mars et al., 2020).

In the Birmingham dataset we observed that baseline alpha diversity correlates negatively with clinical indices of disease severity. Indeed, lower alpha diversity at baseline is associated with a greater likelihood of going on to require AT. In UC, patients who do not respond to 5-aminosalicylates show a significant reduction in

alpha diversity, in additional to multiple taxonomic changes at genus level. The microbial metabolism of 5-ASAs has already been demonstrated to have a negative impact on clinical efficacy of the drug (Mehta et al., 2023) and this shift in the microbiota seen with IBD may be a key driver to therapy failure.

5.4.2 Who are the key players within the microbial community at IBD onset?

Recent studies suggest the oral microbiome may also contribute to IBD using what is known as the oral-gut axis (Wang et al., 2024). Oral-gut transmission is considered to occur regularly, but markedly increase during disease (Schmidt et al., 2019, Kageyama et al., 2023). We confirmed consistent increases in bacteria typical of the oral cavity in the gut of both CD and UC patients. Additionally, we have now shown this across samples of diverse geographic origin and age. Whilst the oral cavity may serve as a reservoir for pathobionts, further work is required to understand the process driving the migration and apparent colonisation of these genera within the gut. The loss of SCFA producing genera, also shown in these patients, has previously been associated with losses of secondary bile acids (Franzosa et al., 2018). In inception studies undertaking metabolomic analyses these patterns were observed with increases in primary and loss of secondary bile acids (Lloyd-Price et al., 2019, Wang Y et al., 2021, Diederen et al., 2020). Consequently, degraded mucosal barrier function may further allow the colonisation of oral pathobionts.

Within the Birmingham dataset, I have been able to link significant baseline enrichment of oral type bacteria with future adverse treatment responses, particularly with regard to *Fusobacterium, Haemophilus, Porphyromonas, Peptostreptococcus*

and *Gemella.* In addition to these genera, UC patients who did not sustain responses to Mesalazine harboured significant enrichment of *Veillonella* and *Aggregatibacter.* It is known that *Haemophilus*, alongside *Veillonella*, can provoke maturation of dendritic cells and programme T cell responses following bacterial exposure (Larsen et al., 2012). Engevik et al. (2020) were able to show both in vivo and in vitro, that *Fusobacterium nucleatum* was able to adhere to the mucous layer of colonic epithelial cells and upregulate inflammatory cytokines, triggering inflammatory cascades. Another genus enriched in our 16S data, *Escherichia.Shigella,* has also been shown to be capable of adherence to and invasion of intestinal epithelial cells (Palmela et al., 2018). In their recent study, Liao et al. (2024) argue, based on mouse models and secondary analysis, that the relative increase in oral bacteria is a secondary phenomenon rather than a driving force. The authors view this increased abundance as simply a marker of ongoing transit from the oral cavity alongside depletion of other gut bacteria. This conclusion however has not been demonstrated with prospective data in an IBD setting, nor is it a view shared universally across all parties working in this field (Read et al., 2021).

The Birmingham data provides additional insights regarding SCFA producers. Depletion of *Phascolarctobacterium* was observed. This is an established feature of bowel inflammation both in early IBD (Morgan et al., 2012) and in established disease (Bajer et al., 2017). *Phascolarctobacterium* is felt to have an important functional role via its utilisation of succinate of produce propionate. Depletion of the *Oscillospirales* order including the genus *Oscillibacter* was also noted in our data. *Oscillibacter* is known to produce Butyrate and in some instances utilise glucoronate (Gophna, Konikoff and Nielsen, 2017), which has previously been shown to be

depleted in IBD. Both *Oscillibacter,* and another genus known to reduce cholesterol to coprostanol (Ren et al., 1996), *Eubacterium Coprostanoligenes,* were depleted amongst the UC cohort who went on to require AT.

Notwithstanding the pooled analysis, our Birmingham dataset has its own limitations. Whilst the metagenomics has been used to assess Meta-CYC pathways, the absence of a dedicated metabolomic data to supplement our conclusions is an omission that must be acknowledged. This need to deliver the clinic pragmatically and not delay diagnosis made obtaining stool samples stabilised with a preservation buffer desirable to maximised convenience for patients, but these buffers do not facilitate metabolomic analysis. Another limitation of our work arises from my bioinformatic approach. I have broken many of the analyses down into their component parts using MaAsLin2. Though our dataset is well matched in terms of age and ethnicity, the sex make-up of the cohorts differs, and this has not been factored into all the analyses.

5.4.3 Abundance associations and microbial function from shotgun data

A key species that showed enrichment in association with adverse outcomes through our shotgun abundance and functional data is *Sutterella Wadsworthensis*. The genus *Sutterella* was enriched in UC in our pooled analysis, but only seen in paediatric cohorts. However, the ability to differentiate strains has also been crucial in delineating roles within this analysis. The strain SGB9283 was seen to associate with lower disability at presentation as determined by the IBD disk (L2FC -2.37 FDR 0.03), yet the strain SGB9286 associated with an increased risk of future AT

utilisation. Increased functional metabolic activity from *S. Wadsworthensis* was seen to associate with increased endoscopic severity in UC (2 pathways). These signals would be in keeping with the findings of key early studies including Mottawea et al. (2016) and Schirmer et al. (2018). *Sutterella* is also discussed in a study by Shapiro et al. (2021) where increased IgA coating of *Sutterella* was found in specific individuals but not significantly more in IBD than controls, where it was postulated that particular strains may be more highly immunogenic. Indeed, Sutterella has been linked to the failure of faecal microbial transplantation (FMT) in UC (Paramsothy et al., 2019). It was actively sought and excluded in more recent FMT studies (Haifer et al., 2022).

The importance of several species of *Bacteroides* are highlighted by the functional data, a finding that would otherwise be missed if just evaluating overall abundance. Metabolic activity from *Bacteroides vulgatus, uniformis* and *dorei* all reduced in those who had worse endoscopic severity at UC presentation and were more likely to not respond to Mesalazine therapy. Indeed, and important role for *Bacteroides* has been demonstrated across several human and mouse studies. Vatanen et al. (2016) demonstrated the *Bacteroides dorei* lipopolysaccharide inhibits the immunostimulatory activity of the *Escherichia coli* Lipopolysaccharide. Further to this, *Bacteroides uniformis* has been shown to alleviate colitis progression in a dextran-sulfate sodium model of colitis in mice, increasing the abundance of *Bifidobacterium* and decreasing *Escherichia.shigella*, whilst also modulating bile acid metabolism (Yan et al., 2023).

5.4.4 Novel microbial associations with overall disability at IBD onset

By combining the 16S datasets with the IBD disk data I have demonstrated novel associations between disability and microbial abundance. *Lachnoclostridium* is a *Lachnospiraceae* in which increased abundance has previously been associated with depressive symptoms, postulated to owe to a role in the synthesis of glutamate, butyrate, serotonin and gamma amino butyric acid (Radjabzadeh et al., 2022). Increased abundance has previously been demonstrated in established CD patients (Frau et al., 2021), whilst reduced abundance has been demonstrated in active UC (Zhu et al., 2022). In our cohort, increases in abundance of *Lachnoclostridium* did not significantly associate with endoscopic severity. However, amongst our IBD cohort, increased abundance of *Lachnoclostridium* is significantly associated with higher total IBD disk scores, as well as higher fatigue scores (‘*Energy’* domain).

Whilst these associations are not a demonstration of cause, it does demonstrate that there are significant microbial contributions to psychosocial well-being over and above the changes associated solely with mucosal inflammation.

5.4.5 Future directions

Established bidirectional networks are already described between periodontal inflammation (characterised by oral dysbiosis) and IBD (Byrd and Gulati, 2021). Recent mechanistic work has further highlighted the importance of these interactions in the modulation of IBD, with some of these oral pathobionts being shown to prompt adaptive immune responses (Atarashi et al., 2017). Exploration of these mechanistic links is in its infancy. To date, one paper has studied the associations between oral

and gut microbiome, alongside their ability to separate IBD from healthy, at paediatric disease onset (Somineni et al., 2021). Another study characterises the oral microbiome of pre-treatment patients with newly diagnosed CD but makes no link to the gut microbiome (Elmaghrawy et al., 2022). No studies explore this in adults. Indeed, no study has explained why in a disease that often presents in childhood where periodontal disease is typically absent, why the oral cavity seems to have such a role in immune priming. Furthermore, if changes in microbial metabolism increase the risk of conventional therapy failure in UC, attempts to correct this could prevent disease progression and enhance 5-aminosalicylate efficacy.

To help answer these questions, I have three objectives:

- Firstly, within this thesis, I will seek associations between the microbial changes described here and the expression of galectins as presented in Chapter 4. In the longer term, we have alongside collaborators in Roche, curated a large mucosal single cell dataset from this cohort (now totalling 1.37 million cells across multiple compartments) and multi-omic integration of these datasets is a key objective.

The latter two are being delivered currently as part of my role in the Oral and Systemic Health BRC theme:

- First, we are collecting linked oral/gut microbiome samples (stool, plaque, saliva and periodontal fluid) from our IBD inception patients. To date we have undertaken pilot metagenomic sequencing on 30 (10 CD, 10 UC, 10 SC).
- Secondly, we are aiming to deliver a randomised control trial of FMT early in the disease course of UC. We have shown clear perturbations in the microbial

community at UC onset, with several associated with disease activity and outcome. Given these changes are present from disease outset, intervention should also be undertaken promptly to try to restore a ‘healthier’ microbial community before inflammatory pathways are ingrained. Increased efficacy from earlier intervention with FMT is already supported by systematic review evidence (Rees et al., 2022). We have submitted a grant to this effect and if successful I hope it will form a key focus of my early consultant career.

5.5 Conclusions

The integrated analysis presented here is of the pre-treatment microbiome in IBD to date. Through this I have demonstrated key microbial perturbations at IBD onset, including those that are present across IBD subtypes, sample types and patient age groups. Both CD and UC display enrichment of microbiota typical of the oral cavity at disease onset. In our Birmingham dataset, longitudinal follow up has allowed us to link these genera with the failure to sustain responses conventional mesalazine therapy and steroid induction in UC. Alpha diversity remains important, though despite clear associations with disease severity, significant differences in diversity are not consistently demonstrated when comparing IBD subtypes with SC.

I will further explore these changes through an ambitious programme of ongoing work characterising the oral and gut microbiome, integrating microbial readouts with mucosal single cell dataset and through a study of microbiome intervention using FMT in recently diagnosed UC.

CHAPTER 6: Identifying interactions between galectins and the gut microbiome at IBD onset

6.1 Abstract

Introduction

β -galactoside binding lectins (galectins) are a family of glycan binding proteins with key roles in mediating inflammatory pathways and host-microbial interactions. In chapters 4 and 5, our analyses linked serum GAL-9 levels and individual microbial taxa to treatment responses at IBD onset. The relationships between galectins and gut microbiota have never been assessed in IBD.

Methods

IBD and SC patients were recruited to research pre-treatment. Serum levels of GAL-1, -3 and -9 were measured by ELISA. Faecal samples were subjected 16S rRNA and shotgun metagenomic sequencing. 16S analyses were performed with QIIME2 and metagenomics with HUMAnN3. Integrated galectin / microbial analysis was undertaken using MaAsLin2 and Phyloseq.

Results

GAL-1 positively correlated with 16S derived Shannon alpha diversity in UC only (n=34 r_s=0.39 p=0.02), whilst GAL-9 correlated inversely with alpha diversity in CD (n=41 r_s= -0.31 p=0.05). No significant correlations were observed for alpha diversity amongst SC. In IBD patients with 16S data, GAL-9 levels correlated with enrichment of *Ruminococcus gnavus* (FDR 0.16) and depletion of SCFA producers such as *Agathobacter* (FDR 0.01) and *Fusicatenibacter* (FDR 0.19). Differential abundance of these genera has been independently linked to treatment responses. These associations were not observed in SC.

A binomial predictive model was developed using Hb, CRP, WCC, IBD Disk and FCP to predict future need for AT within 2 years of diagnosis. This model was significant (McFadden's pseudoR^2 = 0.304 p<.001) and predictive of AT requirement (AUC 0.859). However, adding GAL-9 levels and 16S derived abundance of *Fusicatenibacter, Agathobacter* and *Fusobacterium* (given its links to future disease phenotype) increased the strength (McFadden's pseudoR^2 0.499 p<.001) and predictive ability (AUC 0.922) of the model. Abundance of *Fusicatenibacter* was the strongest predictor in this model (Wald stat 4.6 p=0.03), with GAL-9 level third strongest (Wald stat 2.5 p=0.12) behind FCP (Wald stat 3.7 p=0.06).

In the shotgun dataset, associations with GAL-9 were weaker. However, both GAL-1 and GAL-3 significantly correlated with microbial metabolic activity. When correcting the analysis for baseline mucosal inflammation, GAL-9 levels were positively associated with the metabolic activity of the pathobiont *Sutterella Wadsworthensis.*

Conclusions

Serum GAL-1, -3 and -9 levels correlate with abundance and functional metabolic activity of several key microbiota. GAL-1 associates positively with alpha diversity in UC, whilst GAL-9 inversely correlates with alpha diversity in CD. Across IBD, enrichment of *Ruminococcus Gnavus* and depletion of key SCFA producers, including *Fusicatenibacter,* are seen alongside higher GAL-9 levels. Using predictive modelling, integration of baseline clinical, microbial and galectin data accurately predicted future AT use. If validated on a larger scale, this could make patient stratification and therapeutic personalisation more achievable at IBD diagnosis.

6.2 Introduction

The published mechanistic work identifying the role of GAL-3 and GAL-9 in host-pathogen interactions is explored in the first chapter. However, these links are also seen with GAL-1. In mouse models, GAL-1 has been shown to modulate dendritic cells and enhance immune tolerance, thus suppressing autoimmunity (Ilarregui et al., 2009). Furthermore, GAL-1 has been shown to directly contribute to bacterial autophagy (Lin et al., 2020). Coupled with the greater association between GAL-1 and mucosal measures of inflammation, particularly in CD, potential interactions between any of these galectins and specific microbiota could be highly relevant in the context of IBD onset. Despite this, relationships between microbial communities in IBD have never been linked to galectin expression, and certainly not prior to the initiation of pharmacologic therapy.

6.3 Results

In total, 89 patients (41 CD, 34 UC, 14 SC) had paired 16S and GAL-1 or GAL-3 levels at baseline, whilst it was 90 for GAL-9 (41 CD, 34 UC, 15 SC). Regarding the Shotgun data, 70 IBD (39 CD, 31 UC) patients had paired galectin levels.

6.3.1 Associations between serum galectin levels and microbial diversity

Across the whole 16S cohort, no significant associations were seen between measured galectins and alpha diversity (Shannon). When split into diagnostic subgroups, GAL-1 demonstrated a significant positive correlation with Shannon diversity in UC (n=34 r_s=0.39 p=0.02) but not CD (n=41 r_s=0.16 p=0.31). GAL-9

showed a weak inverse correlation with Shannon diversity in CD (n=41 r_s=-0.31 p=0.05) but not UC (n=34 r_s=0.01 p=0.96). No significant correlations were observed with SC across all galectins (n=14, GAL-1 r_s=-0.08 p=0.79, GAL-3 r_s=-19 p=0.53, GAL-9 r_s=-0.004 p=0.99).

This pattern was replicated in the Shotgun data. Here, significant positive associations were seen between GAL-1 and Shannon diversity in UC (n=31 r_s=0.57 p<.001) but not CD (n=39 r_s=0.06 p=0.72). GAL-9 levels trended towards a negative association with Shannon in CD, but surprisingly a positive association in UC. Both were outside of statistical significance (UC n=31 r_s=0.29 p=0.11, CD n=39 r_s=-0.23 p=0.17). No meaningful correlations were identified with GAL-3.

6.3.2 Linking microbial abundance with serum galectins – the 16S dataset

Given the low numbers in the longitudinal dataset, analyses have focussed on the integration of baseline galectin results with microbial abundance data. GAL-1 showed the closest associations with alpha diversity in UC. Despite this, no significant associations with microbial abundance were identified when UC patients were analysed in isolation using MaAsLin2. When IBD subtypes were grouped together, small but significant associations were observed, with increases in *Catenibacterium* (L2FC 0.61 FDR 0.02) and decreases in *Bacteroides* (L2FC -0.76 FDR 0.04) observed with increasing serum levels of GAL-1.

For GAL-3, the opposite was observed, with no significant associations across grouped IBD, but unique findings for each subtype. In CD, increases in GAL-3 correlated positively with abundance of *Eisenbergiella* (L2FC 0.57 FDR 0.007) and *Marvinbryantia* (L2FC 0.47 FDR 0.01). In UC patients, increases in GAL-3 inversely correlated with *Anaerostipes* (L2FC -1.09 FDR 0.16) and positively correlated with *Coprobacter* (L2FC 1.50 FDR 0.16).

The most striking signals within the data were seen regarding the associations with GAL-9. Across the grouped IBD cohort, increases in GAL-9 associated with enrichment of *Ruminococcus gnavus* (L2FC 0.91 FDR 0.16). Higher GAL-9 was also observed alongside lower levels of several SCFA producers including *Agathobacter* (L2FC -1.29 FDR 0.01), *Coprococcus* (L2FC -0.86 FDR 0.16)*, Eubacterium eligens* (L2FC -0.63 FDR 0.16) and *Fusicatenibacter* (L2FC -0.64 FDR 0.19). On subgroup analysis, most of this signal came from UC, with no significant associations for CD. This is somewhat surprising given the stronger association with reduced alpha diversity is observed in CD. The data, outside of FDR threshold, does demonstrate broad depletion of multiple *Clostridia* including *Butyricimonas, Agathobacter, Fusicatenibacter and Faecalibacterium* in CD (but all FDR 0.44). The significant differences observed in UC are shown in ***Figure 6-1***.

Across analyses amongst SC, no significant associations were observed with GAL-1 or GAL-3. Increased GAL-9 levels were seen in those with enrichment of *Sellimonas,* a member of the *Lachnospiraceae* family, but no other microbial taxa.

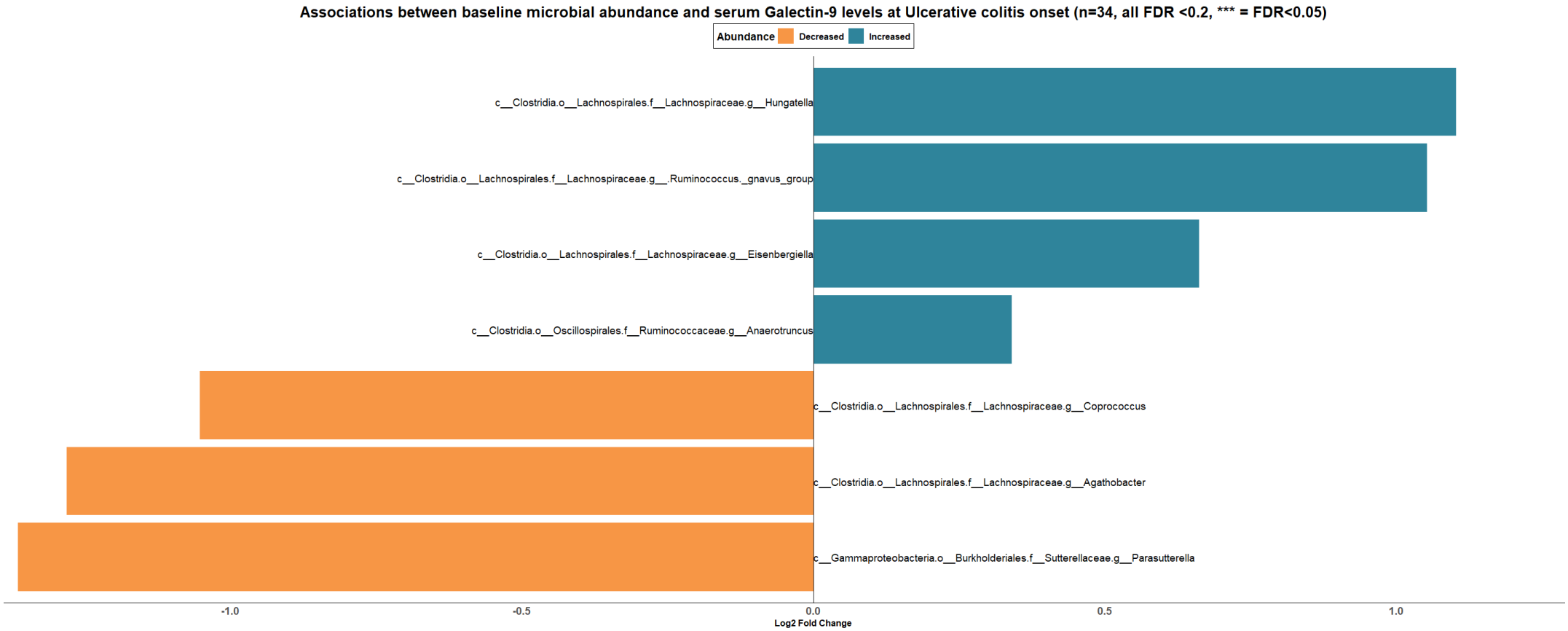


Figure 6 - 1: The association between the abundance of microbial genera in faecal samples and serum GAL-9 levels in pre-treatment UC patients

Across a 16S UC dataset, 34 patients had paired GAL-9 ELISA data and 16S sequencing output. A significant positive correlation between GAL-9 and *Hungatella* (L2FC 1.1 FDR 0.03) and *Ruminococcus Gnavus* (L2FC 1.05 FDR 0.19) amongst others. Significant negative correlation was seen between GAL-9 and *Agathobacter* (L2FC -1.28 FDR 0.06), *Coprococcus* (L2FC -1.05 FDR 0.19) and *Parasutterella* (L2FC -1.36 FDR 0.19).

6.3.2 Results: Using Phyloseq to search for relative associations across the 16S data encompassing baseline factors, treatment outcomes and galectin levels

To build on the signals described earlier, I have utilised Phyloseq to factor in demographics (age and sex), baseline indices (Hb, WCC, CRP and FCP) and outcomes (failure of conventional therapies and commencement of AT). The objective of this approach was to see if significant associations with galectin levels remain when the factoring in the relative differences across these indices, and how this interplay can be linked with treatment outcomes. This analysis is displayed in a heatmap in ***Figure 6-2****,* with the original outputs linked in **Appendix E**. Rather than L2FC, coefficients here are a spearman correlation with an FDR adjusted p value.

GAL-9 levels are still seen to negatively associate with the abundance of SCFA acid producers including *Fusicatenibacter*, *Coprococcus* and *Agathobacter.* Their depletion is also seen to associate with the future failure conventional therapies and the initiation of AT across IBD subtypes. Concordant signals for GAL-1 and GAL-3 are not identified during this analysis.

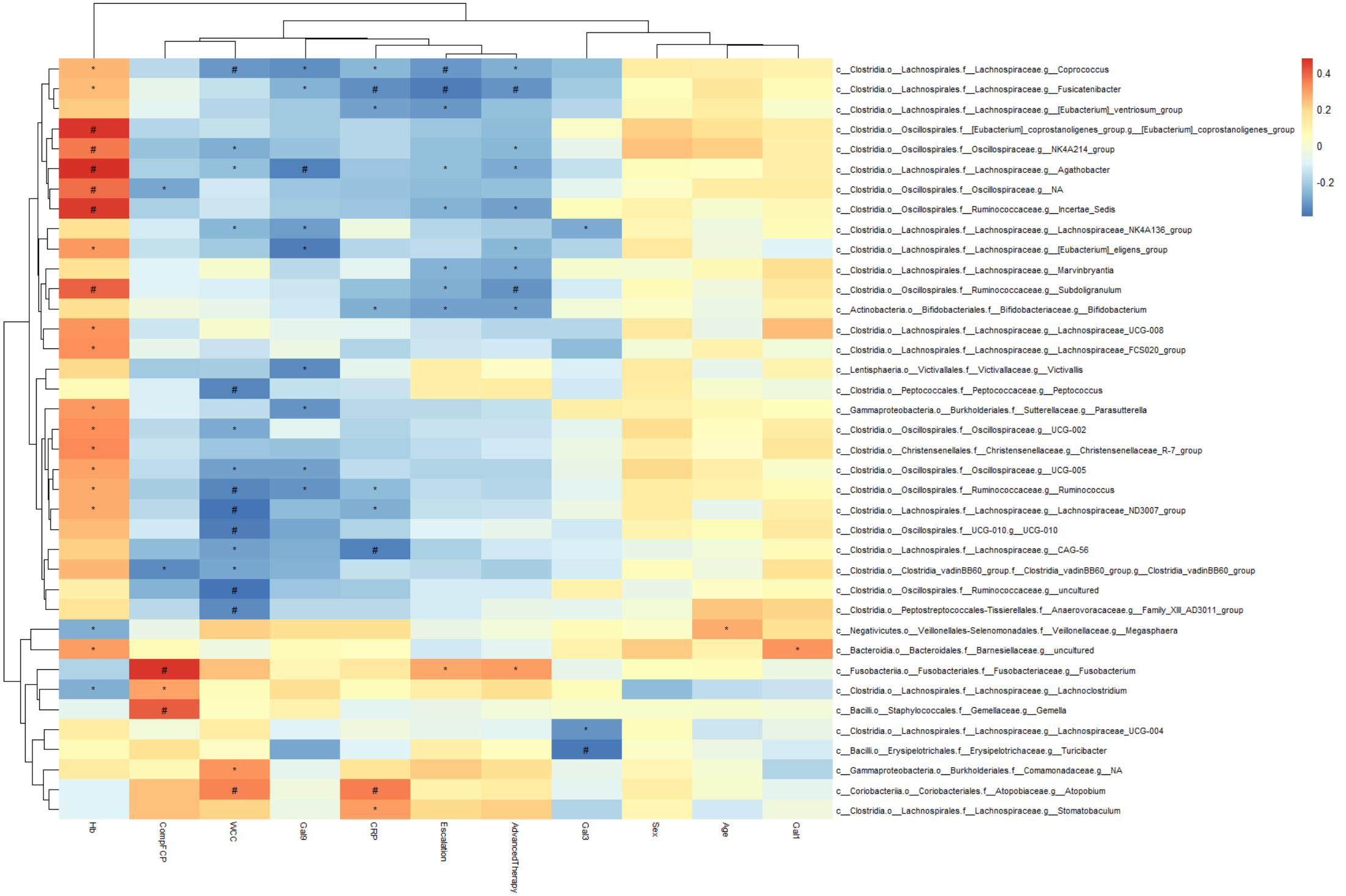

c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Coprococcus
c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Fusicatenibacter
c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__[Eubacterium]_ventriosum_group
c__Clostridia.o__Oscillospirales.f__[Eubacterium]_coprostanoligenes_group.g__[Eubacterium]_coprostanoligenes_group
c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__NK4A214_group
c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Agathobacter
c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__NA
c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__Incertae_Sedis
c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Lachnospiraceae_NK4A136_group
c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__[Eubacterium]_eligens_group
c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Marvinbryantia
c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__Subdoligranulum
c__Actinobacteria.o__Bifidobacteriales.f__Bifidobacteriaceae.g__Bifidobacterium
c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Lachnospiraceae_UCG-008
c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Lachnospiraceae_FCS020_group
c__Lentisphaeria.o__Victivallales.f__Victivallaceae.g__Victivallis
c__Clostridia.o__Peptococcales.f__Peptococcaceae.g__Peptococcus
c__Gammaproteobacteria.o__Burkholderiales.f__Sutterellaceae.g__Parasutterella
c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__UCG-002
c__Clostridia.o__Christensenellales.f__Christensenellaceae.g__Christensenellaceae_R-7_group
c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__UCG-005
c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__Ruminococcus
c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Lachnospiraceae_ND3007_group
c__Clostridia.o__Oscillospirales.f__UCG-010.g__UCG-010
c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__CAG-56
c__Clostridia.o__Clostridia_vadinBB60_group.f__Clostridia_vadinBB60_group.g__Clostridia_vadinBB60_group
c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__uncultured
c__Clostridia.o__Peptostreptococcales-Tissierellales.f__Anaerovoracaceae.g__Family_XIII_AD3011_group
c__Negativicutes.o__Veillonellales-Selenomonadales.f__Veillonellaceae.g__Megasphaera
c__Bacteroidia.o__Bacteroidales.f__Barnesiellaceae.g__uncultured
c__Fusobacteriia.o__Fusobacteriales.f__Fusobacteriaceae.g__Fusobacterium
c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Lachnoclostridium
c__Bacilli.o__Staphylococcales.f__Gemellaceae.g__Gemella
c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Lachnospiraceae_UCG-004
c__Bacilli.o__Erysipelotrichales.f__Erysipelotrichaceae.g__Turicibacter
c__Gammaproteobacteria.o__Burkholderiales.f__Comamonadaceae.g__NA
c__Coriobacteriia.o__Coriobacteriales.f__Atopobiaceae.g__Atopobium
c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Stomatobaculum
Hb
CompFCP
WCC
Gal9
CRP
Escalation
AdvancedTherapy
Gal3
Sex
Age
Gal1
0.4
0.2
0
-0.2

Figure 6 - 2: Heatmap developed utilising Phyloseq and pheatmap, displaying relative microbial associations across various demographic, disease severity and treatment outcome parameters, alongside serum levels of GALectin-1, -3 and -9.

This analysis encompasses all IBD patients in the 16S data (n=89, CD=45 UC=34). Focussing predominantly on GAL-9 data, *Fusicatenibacter* (Coef -0.28 p_{adj} 0.04), *Coprococcus* (coef -0.33 p_{adj} 0.01) and *Agathobacter (Coef -0.36* p_{adj} 0.008) negatively associated with GAL-9 levels. Depletion of these genera is also seen to associated with a future need for AT. Only genera with at least one significant association are shown on the heatmap.

6.3.3 Results: Additional signals from the shotgun dataset

Following the same approach described earlier, I sought to further interrogate these patterns in our shotgun metagenomic data to track differences to species and strain level, whilst also analysing microbial function.

Significant associations are limited throughout the shotgun dataset. Regarding GAL-1, in the overall IBD cohort, only weak positive associations were seen with the abundance of an unnamed species of *Ruminococcaceae (GGB9614 SGB15049* L2FC 0.44 FDR 0.17) and a *Bacteroidete* (GGB1420 SGB 1957 L2FC 0.46 FDR 0.17). For CD, increased abundance of *Bacteroides eggerthii (SGB1829* L2FC 0.69 FDR 0.09) is seen with increasing GAL-1.

Stronger associations are observed between microbial function and GAL-1 levels. Amongst grouped IBD subtypes, higher GAL-1 levels were observed alongside increased activity from *Alistipes putredinis* across 21 metabolic pathways. Bacteroides is also associated here with increased activity from *Bacteroides vulgatus* (3 pathways), *uniformis* (1 pathway) and *massiliensis* (1 pathway). On subgroup analysis, the majority of the *Alistipes putredinis* is derived from UC patients where 24 pathways show a positive correlation with GAL-1. In CD, a separate signal is observed, with positive associations observed with *Bacteroides eggerthii* (45 pathways) and *Parabacteroides distasonis* (27 pathways).

For GAL-3, a single significant association was observed with an unclassified *Clostridia (SGB15374).* On subgroup analysis, this difference is driven by the CD cohort, where a *Ruminococcaceae* (*GGB9699 SGB15216*) is also differentially abundant. As with GAL-1, I was able to identify significantly more significant associations with metabolic function. Across IBD, higher GAL-3 levels associated with increased activity of *Parabacteroides distasonis* across 33 metabolic pathways. This signal is entirely derived from those with CD with differential activity seen in 45 pathways relating to *Parabacteroides distasonis,* with further positive associations seen with the metabolic activity of *Bacteroides caccae* (12 pathways).

A similar approach was taken to the analysis of GAL-9. The only observed significant association was identified across grouped IBD subtypes. Increased GAL-9 levels correlated positively with abundance of *Candidatus gastranaerophilales bacterium* (L2FC 0.73 FDR 0.02). No signals were observed amongst IBD subtypes. If the analysis was corrected for mucosal inflammation at the time of sampling ("FCAL2"), a weak positive association was seen between GAL-9 and *Sutterella Wadsworthensis SGB9286* (L2FC 1.52 FDR 0.22), but this sat outside of our defined significance threshold. A similar pattern was identified regarding metabolic activity. No significant signals were observed relating to GAL-9 across either grouped IBD or IBD subtypes when performing unadjusted analysis. However, if correction for mucosal inflammation was undertaken, again using "FCAL2", increased activity of *Bacteroides Massiliensis* was observed across 5 metabolic pathways, with increased activity from *Sutterella Wadsworthensis* in one pathway (inosine 5 phosphate degradation, L2FC 0.56 FDR 0.07).

6.3.4 Results: Can modelling this data enable earlier identification of future disease behaviour?

Within the clinical data, galectin ELISAs and microbiome analyses there are several differences that it may be possible to leverage to predict treatment outcomes. This is particularly for the early recognition of those going on to require AT. Given the larger dataset, lower cost and greater scalability, I have chosen to utilise the 16S data in this attempt at modelling. I have developed a simple binomial regression model based on clinical indices that are relevant and available at first presentation across disease subtypes (Haemoglobin, CRP, WCC, FCP and IBD Disk) and supplemented these with GAL-9 levels and absolute abundance of key microbial genera that either link to GAL-9 levels, treatment outcomes or both (*Fusicatenibacter, Agathobacter* and *Fusobacterium).* Only individuals with complete data were included in the model, which left 59 IBD patients (CD 33, UC 26) of whom 27 required escalation to AT after the failure of conventional treatment (CD 21, UC 6). The overall model was significant (McFadden's PseudoR^2 = 0.499, p <.001). Using the Wald test, abundance of *Fusicatenibacter* was the strongest predictor within the model (Wald statistic 4.61 p=0.032), followed by FCP (Wald statistic 3.66 p=0.056) and GAL-9 levels (Wald statistic 2.48 p=0.11). The overall model carried a sensitivity of 0.81, specificity of 0.82 and AUC of 0.922. This equivalent model, based on only the clinical parameters was inferior across overall model statistics and predictive capacity in the same patient cohort (McFadden's PseudoR^2 = 0.304 p<.001, Sensitivity 0.80, Specificity 0.62, AUC 0.859). The outputs from the model with microbial and GAL-9 data included are displayed in ***Figure 6-3***.

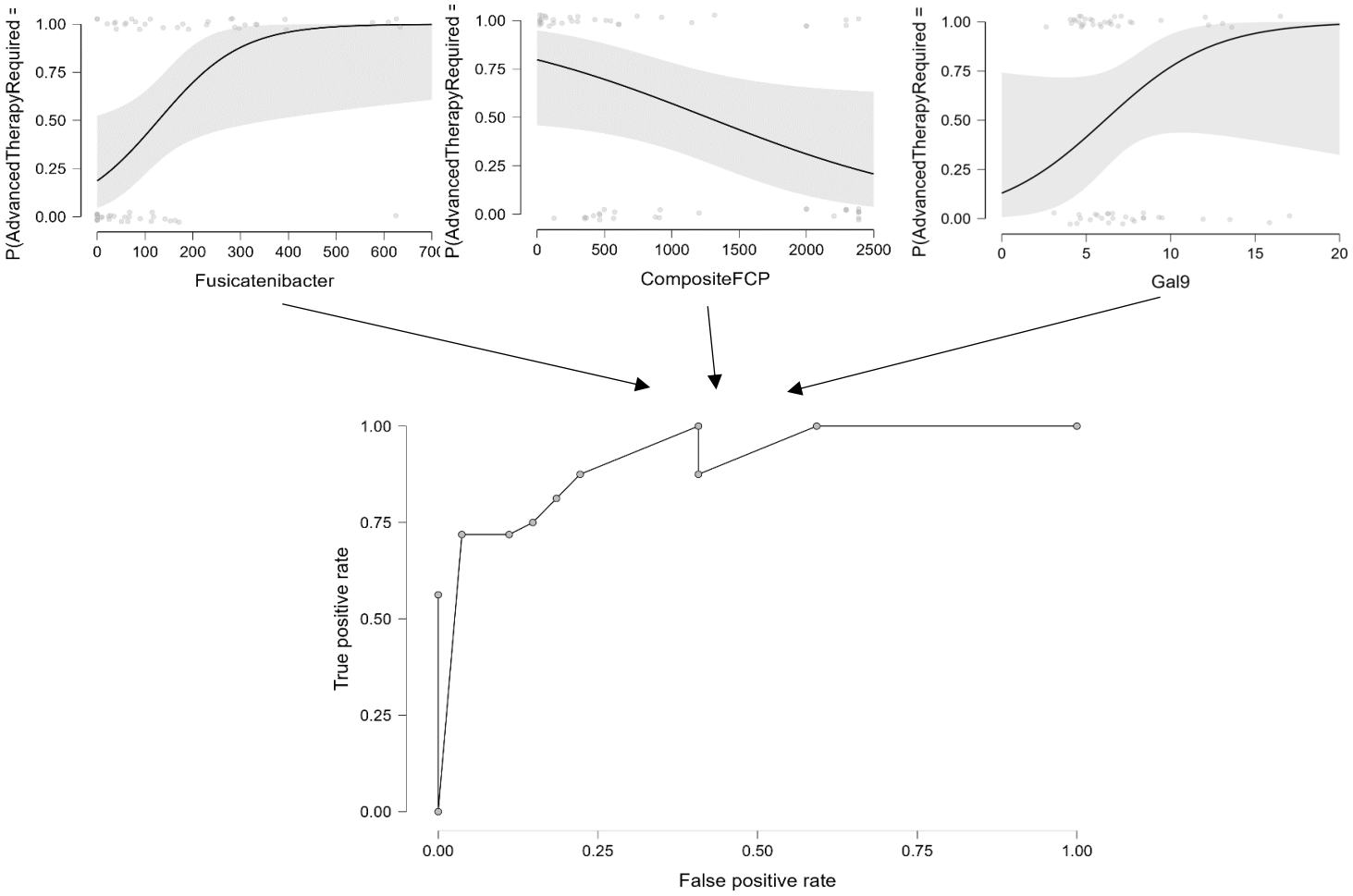


Figure 6 - 3 Inferential plots showing 95% Confidence intervals for the top 3 model contributors and an overall Receiver Operating Characteristic curve for predictive ability of a binomial regression model to couple baseline clinical indices, faecal microbial abundance and serum GAL-9 levels to predict future need for AT in IBD. 'AdvancedTherapyRequired' level 'No' as class 1.

These evaluations were only undertaken in those patients within the 16S sequencing dataset who had GAL-9 levels determined by ELISA, longitudinal outcome data and relevant clinical indices available including an IBD disk. This meant the cohort was reduced to 59 patients (33 CD, 26 UC). Of these, 27 did not obtain an optimal response to conventional therapies and progressed to AT during the follow up period (CD 21, UC 6). The overall regression model was significant (McFadden's PseudoR^2 0.499 p<0.001) and carried a sensitivity of 0.813, specificity of 0.815 and AUC 0.922. The strongest predictor was *Fusicatenibacter* (Wald stat 4.61 p=0.032), followed by faecal calprotectin (Wald stat 3.66 p=0.056) and GAL-9 level (Wald stat 2.48 p=0.115). This outperformed a model based purely on the clinical parameters described above (McFadden's PseudoR^2 = 0.304 p<.001, Sensitivity 0.80, Specificity 0.62, AUC 0.859).

6.6 Discussion

Interpretation of the associations demonstrated between galectin levels and microbial abundance is made challenging by a relatively small cohort with contrasting readouts from the various datasets acquired. Furthermore, the data presented only allows for the establishment of association, without inference of functional relationship. The being said, this is the first attempt to establish these associations in IBD, despite the roles that galectins are established to have in the mediation of immune responses and host microbe interactions. The early signals are intriguing and would warrant validation on a larger scale.

6.6.1 The interplay of GAL-9 and key microbial species

The microbial correlations associated with GAL-9 levels are of great interest. I have already explored the established roles of GAL-9 in microbial engagement / opsonisation, cellular signalling and T-cell regulation. From the analyses of the galectin ELISA data in isolation, presented in Chapter 4 (section 4.4.6), I have established that increased GAL-9 levels at baseline are associated with more severe disease and a risk of conventional therapies failing to obtain control of the IBD, particularly regarding UC. The association of increases in GAL-9 with depletion of SCFA producing bacteria, particularly in UC, is of relevance. For example, in the relative abundance heatmap we see *Agathobacter, Fusicatenibacter and Coprococcus* significantly reduced in those with higher GAL-9 levels. Those patients with greater depletion are more likely to go on to require an AT. Alongside this, GAL-9 levels positively relate with *Ruminococcus gnavus,* a genus previously associated

with adverse treatment outcomes (Schirmer et al., 2018) and an increased risk of surgery for CD patients within the Birmingham cohort.

These specific taxa changes are not replicated in the higher resolution metagenomic data, though if we correct for mucosal inflammation at the time of sampling, serum GAL-9 is shown to associate with increased abundance and metabolic activity of *Sutterella Wadsworthensis*. This association is seen at strain level, with the same strain SGB9286 also being more prevalent in those going on to require AT. This conclusion is less clear cut, particularly given the higher FDR thresholds observed and the additional caveats placed on the analysis. It is a signal that undoubtedly would require further evaluation in a cohort of greater size and a dataset of greater sequencing depth.

6.6.2 Understanding novel microbial interactions with GAL-1 and GAL-3

As described in the existing literature, GAL-1 levels were again more closely linked to mucosal inflammation, with serum levels correlating with faecal calprotectin. Within our microbial datasets, we also observe significant associations between GAL-1 and the bacterial alpha diversity measured in stool. Within the 16S dataset, increased pre-treatment GAL-1 levels were seen to associate with a reduction in the abundance of the Bacteroides genus, with increases in Catenibacterium. These signals are not replicated in the shotgun dataset. However, this dataset does highlight a strong association between GAL-1 and the metabolic activity of several microbial taxa. In UC patients, increasing GAL-1 levels associate with increased metabolic activity from *Alistipes putridenis*, a microbe previously associated with

health (de Meij et al., 2018). In CD, increased activity is seen across a multitude of pathways with contributions from species including *Bacteroides eggerthii and Parabacteroides distasonis.* Very few associations could be drawn between microbial abundance and GAL-3 levels. Despite this, in CD there were a significant number of associations with bacterial metabolic activity, with positive associations observed between GAL-3 levels and increased activity from *Parabacteroides distasonis* and *Bacteroides caccae.* Strains of *Parabacteroides distasonis* have been shown to attenuate colitis in mouse models (Kverka et al., 2010) and exert an anti-inflammatory action in vitro, a process mediated via IL-8 (Hiippala et al., 2020). We have shown in our ELISA data that amongst those with UC, higher GAL-3 levels are seen in those with the mildest disease. It is plausible that some part of this could owe to consequent microbial interactions. At this stage this is too great a leap to make, a case made stronger by most of these microbial interactions coming from CD, where clearly associations with serum GAL-3 levels and disease severity have not been demonstrated, either by my data or others.

6.6.3 Does our modelling represent a tool with clinical utility?

Whilst in the present clinical environment adding abundance counts to such models may seem somewhat arbitrary, it does highlight the additional predictive capacity that this easily scalable technology could provide to help clinicians stratify patients from the point of diagnosis. A model based on clinically validated tools well established in day-to-day practice was significantly improved by serum and faecal microbial indices that are based on low cost and scalable technology. Indeed, the microbial abundance of *Fusicatenibacter* was able to contribute more to our model than any of

the well-established clinical indices. Though our cohort is relatively small for seeking to undertake modelling and draw broad conclusions, it does suggest that future exploration in a larger cohort might contribution to our efforts to bring personalised medicine closer to our patients.

6.6.3 Future directions

Our data highlights the close interplay between serum galectins and the abundance of highly relevant gut microbiota. Nonetheless, the data is derived from faecal and serum samples, not capturing what is taking place at the mucosal interface. As part of our collaboration with F. Hoffman La Roche, we have been collecting and processing gastrointestinal mucosal biopsies for single cell sequencing. Integration of these datasets, both regarding cell populations but also cellular gene expression (such as those responsible for the expression of galectins like LGALS1 and LGALS9) may allow us to better understand the relevance of the associations I have described. In doing so, we may be better placed to identify if the described changes have a relevant role to play in dysregulated host-immune interactions at the mucosal interface and how they might relate to treatment outcome.

Whilst we have used the metagenomic data to assess the functional capacity of different microbiota, we have not undertaken any direct assessment of the metabolome, another cornerstone in the pathophysiology of IBD, and one that is intricately linked to the microbiome. Our chosen stool collection kits with buffer allowed delayed collection to prioritise microbiome analyses, but sadly preclude undertaking reliable metabolomic analysis. Whilst using recently developed LC-MS

metabolic profiling on serum samples was considered, it was not practical financially, logistically or in the context of analyses that were already planned for our samples. As our research collaborations move forwards, we plan to prospectively evaluate the metabolome alongside our microbiome analyses.

Finally, it is also acknowledged that the read depth of our metagenomic data, when considering the volume of host material sequenced, would have allowed more detailed analyses if undertaken to a greater depth. At the time of sequencing, the funding available did not allow for this to be undertaken. Through additional funding received as part of our NIHR Biomedical research centre renewal, we are now able to undertake sequencing to 50 million reads for all samples. This may allow us to dig further into species, strain and functional differences within our data.

6.7 Conclusions

Serum GAL-1, -3 and -9 levels correlate with abundance and functional metabolic activity of several key microbiota. GAL-1 positively associates with overall alpha diversity in UC, whilst increases in GAL-9 negatively correlate with alpha diversity in CD. GAL-9 levels positively associate with *Ruminococcus gnavus* in UC, and negatively associate with genera such as *Fusicatenibacter* that when depleted are independently associated with early progression to AT. Regression modelling using GAL-9 levels and microbial data suggests that these indices could complement clinical parameters to facilitate earlier and more accurate risk stratification in IBD.

Data summary and concluding remarks

This thesis presents an integrated approach to improving early disease care in IBD. I have concentrated on clinical approaches to facilitate earlier diagnosis and holistic assessment of factors including psychological disease. I have supplemented this with novel serum and microbial markers that could, when applied correctly, aid with the rapid identification of both disease phenotype and likely treatment responses. If applied successfully, the desired result would be more consistent and accurate individualised risk stratification of IBD patients with consequent early personalised management. I hope to use the basic science work presented here to underpin future studies targeting microbiome intervention early in IBD, particularly for UC.

After exploring the existing literature and subsequent methodologies in Chapter 1 and 2**,** Chapters 3 to 6 explore the clinical impacts and original data that I have generated through this thesis. There are multiple different analyses and approaches presented, and patient contribution is not consistent across each of these analyses. Before the core findings of each chapter are summarised I have developed a figure to help give an overview of patient flow through each of our studies **(*Figure 7 – 1*)**.

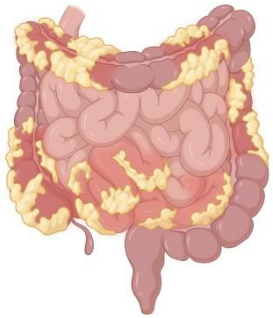
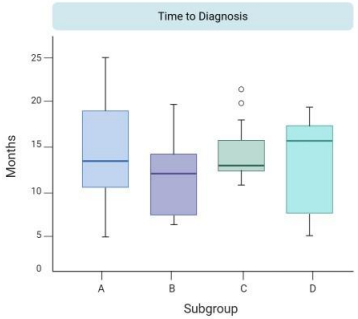
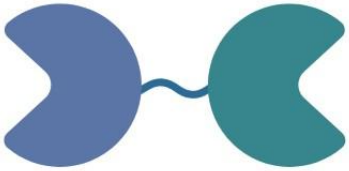
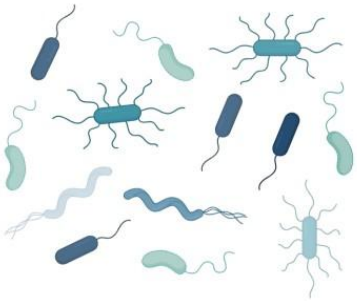
Inception throughput	Clinical data	Galectins	Microbiome	Integration
				
<u>Total patients seen</u> Overall: **762*** Crohn's: **208** UC: **215** Non-IBD: **339**	<u>IBD Modelling</u> Calprotectin: **672** Symptom modelling: **670** Combined modelling: **592** <u>IBD Disk**</u> Overall: **305** Crohn's: **95** UC: **82** Non IBD: **128**	<u>Patients with galectin data</u> <u>Pilot***</u> Overall: **74** Crohn's: **18** UC: **34** Healthy: **22** <u>Inception</u> Overall: **188** Crohn's: **69** UC: **59** Symptomatic non-IBD: **21** Healthy: **39**	<u>Patients with microbiome data</u> <u>16S</u> Overall: **145** Crohn's: **53** UC: **40** Symptomatic non IBD: **52** <u>Shotgun Metagenomics</u> Overall: **78** Crohn's: **45** UC: **33**	<u>Integrated datasets</u> <u>16S + galectins</u> Overall: **90** Crohn's: **41** UC: **34** Symptomatic non IBD: **15** <u>Shotgun + galectins</u> Overall: **70** Crohn's: **39** UC: **31**

Figure 7 - 1: Patient flow and contribution to analyses presented throughout this thesis

Total patient throughput via the Inception pathway, data contribution to clinical analyses, modelling and subsequent serum and microbiome analyses.* Only those who had a final diagnosis at the time of analysis. ** Analyses integrating 16S microbiome and IBD Disk data are derived from 42 patients with Crohn's, 31 with UC and 38 non-IBD. *** Patients in the galectin pilot dataset were not 'inception' IBD patients and were established diagnoses previously recruited.

Returning now to the individual sections, **Chapter 3** explores the considerable service level impact we were able to achieve through redesigning our diagnostic pathway for patients with IBD. With the additional resource, obtained through integration with research, it was possible to undertake dedicated early triage of referrals with prompt identification of those in need. We were able to take an average wait for review of urgent new patients from 84 days to 30 days, with this falling as low as 14 days once our team were aware of the referral. Despite strain on endoscopy resources, we were able to see, diagnose and treat IBD patients within 66% of the time taken for other urgent referrals to receive their first outpatient appointment. This applied regardless of whether the patient participated in the proposed research. Despite this transformation of secondary care pathways, patients with CD still carry significant symptoms for a disproportionately long time prior to referral and diagnosis. To provide better tools to both primary and secondary care physicians making decisions regarding IBD referral and diagnosis, I have undertaken a detailed evaluation of the efficacy of FCP measurement when it is utilised as is currently typical. I have subsequently explored how this could be improved. For the first time, I have characterised the predictive ability of individual symptoms and symptom complexes in a cohort pre-selected by FCP. I have considered how symptoms and FCP could be modelled to allow a radical overhaul of referral pathways, including the offer of direct to patient testing. Finally, I have explored the intimate link between disease activity, psychological symptom burden and early treatment outcomes following IBD diagnosis. I have validated the IBD disk as a PROM to identify clinically significant psychological symptoms, but also to identify patients those with an adverse disease phenotype and increased likelihood of needing inpatient treatment and escalation to AT with the first year after diagnosis.

In **Chapter 4** I have undertaken the first analysis of three β-galactoside binding lectins, or galectins, as a biomarker of disease activity and outcome at IBD onset. I have demonstrated statistically significant increases across UC and CD in GAL-3 (Dunn test, $p_{holm}<0.001$ for CD and UC) and GAL-9 (CD $p_{holm}<.001$, UC $p_{holm}=0.003$) relative to HC. No differences were shown with SC. GAL-1 has shown negative associations with mucosal inflammation in the form of FCP ($r_s= -0.232$ p=0.036) and SES-CD ($r_s= -0.249$ p=0.045), whilst GAL-9 has shown positive associations with serum markers of inflammation (CRP, $r_s=0.375$ p<0.001; WCC, $r_s=0.326$ p<0.001) and key cytokines (TNF $r_s=0.448$ p<0.001; IL-6 $r_s=0.423$ p<0.001). At baseline, higher GAL-9 levels were related with non-response to treatment at 6 months post initiation, particularly within UC patients (p=0.003). Across IBD, higher levels also associated with an increased likelihood of requiring AT within two years of diagnosis (p=0.002). The strength of this difference was put into a regression model to see if GAL-9 levels added additional clinical insight to traditional inflammatory markers including CRP, WCC, platelets, FCP, haemoglobin, ferritin and age. In the binomial model developed, baseline elevations in GAL-9 were shown to be the strongest predictor of treatment non-response at 6 months (omnibus likelihood ratio 10.23 p=0.001). This was replicated in a machine learning decision tree, with GAL-9 again the most important variable. In a binomial model predicting AT utilisation, GAL-9 was the third strongest predictor, behind CRP and ferritin. Across longitudinal sampling, significant reductions were seen in both GAL-3 (Wilcoxon p=0.007) and GAL-9 (Wilcoxon p=0.003) after treatment initiation. GAL-9 levels were significantly influenced by corticosteroid use in a linear regression model (t=2.03 p 0.048). In the small cohort of 10 patients where GAL-9 levels were available at 3 time points, those

with persisting active disease had significant increases in GAL-9 levels (Active n=7 median 7.54, Inactive n=3 median 5.41. Mann Whitney U = 1 p=0.04). Based on our data, whilst galectin levels cannot separate IBD patients from SC, GAL-9 in particular shows a strong association with early treatment responses and baseline elevations could contribute to our ability to identify those in need of earlier more aggressive therapy.

Through **Chapter 5** I describe one of the largest explorations of the pre-treatment microbiome in adult IBD patients to date. With additional bioinformatics support from collaborators, I then integrate these findings with published adult and paediatric metataxonomic datasets to identify the core perturbations that underpin pre-treatment IBD across sample types and patient cohorts. In my own dataset I have been able to utilise both metataxonomic and metagenomic sequencing approaches. Whilst I was not able to demonstrate a significant difference between SC and IBD patients with respect to alpha diversity, I have shown that reductions in alpha diversity at IBD onset associate with an increased future utilisation of AT. This was significant in both CD and UC within the metagenomic dataset (CD median Shannon index 'no AT' 3.02 vs 'AT' 2.34 p=0.018, UC median Shannon 2.66 vs 1.57 p=0.009). In our metataxonomic dataset, UC patients who subsequently did not have a sustained treatment response to Mesalazine (with or without steroid induction) showed an enrichment of phenotypically oral bacteria, including *Fusobacterium* (FDR 0.002), *Haemophilus* (FDR 0.01), *Veillonella* (FDR 0.10) and *Aggregatibacter* (FDR 0.19). These changes were accompanied by depletion of genera including *Fusicatenibacter* (FDR 0.02), *Oscillibacter* (FDR 0.10), and *Bifidobacterium* (FDR 0.13). In our metagenomic dataset, depletion can be traced to strain level with

Fusicatenibacter saccharivorans SGB4874 (FDR 0.19) and *Oscillibacter sp ER4 SGB15254* (FDR 0.19). An additional difference is observed for both the abundance and metabolic activity of *Bacteroides,* in particular *Bacteroides Uniformis* and *Vulgatus.*

In integrating my data from faeces samples with published datasets, I have then been able to challenge the relevance of these differences across broad patient cohorts and sample types. We have been able to generate a sequencing output encompassing 2160 samples, with 1513 from IBD patients. Here, alpha diversity was significantly reduced in both paediatric UC and CD relative to SC but not HC. The opposite was observed in adults, with consistent significant reductions across UC and CD in comparison with HC but not SC. In paediatric patients, signals for reduced alpha diversity were far stronger in mucosal biopsies than in faecal samples. In analyses of beta diversity, faecal samples appear to be more closely related to mucosal biopsies in UC than CD. Despite the vast array of methodologies utilised across these studies, I have identified recurrent themes in differential microbial abundance across these studies and my own. In both adults and children, UC is characterised by an enrichment of bacterial genera typical of the oral cavity. Enrichment of *Aggregatibacter, Haemophilus, Eikenella, Peptostreptococcus, Neisseria* and *Porphyromonas* is detected across faecal samples in adults and children and biopsies in children relative to SC. Whilst this is most prominent in UC, it is also observed in CD with again enrichment of *Aggregatibacter, Neisseria, Peptostreptococcus, Fusobacterium and Eikenella* apparent. Associated depletion of SCFA producers is present across CD and UC, though with depletion of different genera more prominent across each disease subtype.

The differences identified, and the recurrent nature of these across different patient groups does suggest that future interventions targeting the microbiome have a very clear scientific basis early in the disease course. As I move forwards with my career, this is something I would like to interrogate further and a grant application to this end has been submitted, led by my supervisor Prof Iqbal.

Through **Chapter 6** I focus on the integration of our datasets to enhance our understanding of how key galectins, in particular GAL-9 (where roles in host-pathogen interactions and opsonisation have been described) interact with our gut microbiota at IBD onset. Within the metataxonomic dataset, IBD patients demonstrated positive associations between GAL-9 levels and *Ruminococcus Gnavus* alongside negative associations with SCFA producers including *Agathobacter* and *Fusicatenibacter,* both genera earlier shown to be depleted in those going on to require AT. The depletion of these genera was maintained when relative abundance analysis was undertaken across multiple different clinical and biochemical indices using Phyloseq. The changes observed in the 16S data were not replicated in the smaller metagenomic dataset, though significant associations were observed between both GAL-1, -3 and the metabolic activity of several microbial species. If baseline FCP was factored into the analyses, GAL-9 did associate with the pathobiont *Sutterella Wadsworthensis,* both in terms of abundance and metabolic activity.

Several of the associations seen between GAL-9 and microbial genera were with those previously linked to treatment outcomes. I sought to establish if an integrated regression model using these baseline indices could predict future outcomes for IBD patients after treatment initiation. I opted to model GAL-9 levels alongside 16S derived abundance of *Fusicatenibacter, Agathobacter* and *Fusobacterium*. These were modelled with typical indices that would be available during an index outpatient review (Haemoglobin, IBD Disk score, FCP, white cell counts and CRP). The model was able identify those patients that would subsequently go on to require AT with a high degree of accuracy (AUC 0.922).

As I have described through this summary, both galectins, in particular GAL-9, and the microbiome appear to have an important and interconnected role in the processes that drive IBD onset. So far, I have demonstrated association with parameters and potential clinical utilities as a biomarker, but I have yet to demonstrate function or therapeutic potential. As part of our ongoing research programme with Roche, we will explore the galectin data further as these same patients form a part of the largest mucosal single cell atlas that has been curated in pre-treatment IBD to date. This work continues and is being supported by in vitro and functional work.

From a personal perspective, the differential abundance in microbiota and the association of baseline parameters with longitudinal outcomes is a window to a therapeutic opportunity, particularly in UC. Whilst we will continue to grow our inception cohort to increase our statistical power to draw conclusions, our own data

is supported by our pooled analyses of published data. Now then would seem the time to move forwards. Microbiome intervention has not, yet, been tested in an early disease cohort where inflammatory pathways are less well established and multiple lines of treatment non-response aren't already in the patient's history. Faecal microbiota transplantation remains the most well tested intervention in this regard and is the key focus of our current grant application, something I hope will become a key component of the early part of my consultant career.

The work that I have done and the working relationships I have been able to establish, both within the University and with Industry collaborators such as Roche, has enabled me to begin an exciting body of work and put me in a position at the start of my consultant career that I am immensely grateful for. It now rests with me to drive this work forwards and I hope to be able to contribute to our growing understanding of IBD as our work moves forwards.

References

1. Abdill, R.J. et al. (2025) “Integration of 168,000 samples reveals global patterns of the human gut microbiome,” *Cell*, 188, pp. 1100–1118. Available at: https://doi.org/10.1016/j.cell.2024.12.017.
2. Abej, E. *et al.* (2016) “The Utility of Fecal Calprotectin in the Real-World Clinical Care of Patients with Inflammatory Bowel Disease,” *Canadian Journal of Gastroenterology & Hepatology*, 2016, pp. 1–6. Available at: https://doi.org/10.1155/2016/2483261.
3. Acosta, M.V. *et al.* (2011) “Emigration to western industrialized countries: A risk factor for developing inflammatory bowel disease,” *Journal of Crohn’s and Colitis*, 5(6), pp. 566–569. Available at: https://doi.org/10.1016/j.crohns.2011.05.009.
4. Agans, R. et al. (2011) “Distal gut microbiota of adolescent children is different from that of adults,” *FEMS Microbiology Ecology*, 77(2), pp. 404–412. Available at: https://doi.org/10.1111/j.1574-6941.2011.01120.x.
5. Agrawal, M. et al. (2021) “Early life exposures and the risk of inflammatory bowel disease: Systematic review and meta-analyses,” *EClinicalMedicine*, 36, p. 100884. Available at: https://doi.org/10.1016/j.eclinm.2021.100884.
6. Alatab, S. *et al.* (2020) “The global, regional, and national burden of inflammatory bowel disease in 195 countries and territories, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017,” *The Lancet Gastroenterology & Hepatology*, 5(1), pp. 17–30. Available at: https://doi.org/10.1016/s2468-1253(19)30333-4.
7. Ananthakrishnan, A.N. *et al.* (2017) “Environmental triggers in IBD: a review of progress and evidence,” *Nature Reviews Gastroenterology & Hepatology*, 15(1),39–49. Available at: https://doi.org/10.1038/nrgastro.2017.136.
8. Anderson, M.J. (2001) “A new method for non-parametric multivariate analysis of variance,” *Austral Ecology* (2001), 26:32–46. Available at: https://doi.org/10.1111/j.1442-9993.2001.01070.pp.x
9. Arumugam, M. *et al.* (2011) “Enterotypes of the human gut microbiome,” *Nature*, 473(7346), pp. 174–180. Available at: https://doi.org/10.1038/nature09944.
10. Ashton, J.J. *et al.* (2017) “16S sequencing and functional analysis of the fecal microbiome during treatment of newly diagnosed pediatric inflammatory bowel disease,” *Medicine*, 96(26), p. e7347. Available at: https://doi.org/10.1097/md.0000000000007347.
11. Assa, A. *et al.* (2016) “Mucosa-Associated ileal microbiota in New-Onset Pediatric Crohn’s Disease,” *Inflammatory Bowel Diseases*, 22(7), pp. 1533–1539. Available at: https://doi.org/10.1097/mib.0000000000000776.
12. Atarashi, K. *et al.* (2017) “Ectopic colonization of oral bacteria in the intestine drives T_H 1 cell induction and inflammation,” *Science*, 358(6361), pp. 359–365. Available at: https://doi.org/10.1126/science.aan4526.
13. AWARE-IBD Diagnostic Delay Working Group. (2024) “Sources of diagnostic delay for people with Crohn’s disease and ulcerative colitis: Qualitative research study” *PLoS ONE*, 19(6), p. e0301672. Available at: https://doi.org/10.1371/journal.pone.0301672.
14. Bajer, L. *et al.* (2017) “Distinct gut microbiota profiles in patients with primary sclerosing cholangitis and ulcerative colitis,” *World Journal of Gastroenterology*, 23(25), p. 4548. Available at: https://doi.org/10.3748/wjg.v23.i25.4548.

15. Barberio, B. *et al.* (2021) “Prevalence of symptoms of anxiety and depression in patients with inflammatory bowel disease: a systematic review and meta-analysis,” *The Lancet Gastroenterology & Hepatology*, 6(5), pp. 359–370. Available at: https://doi.org/10.1016/s2468-1253(21)00014-5.
16. Barondes, S.H. et al. (1994) “Galectins: a family of animal beta-galactoside-binding lectins,” Cell, 76(4), p. 597-598. Available at: https://doi.org/10.1016/0092-8674(94)90498-7.
17. Basha, O.M. *et al.* (2022) “Impact of gut Microbiome alteration in Ulcerative Colitis patients on disease severity and outcome,” *Clinical and Experimental Medicine* [Preprint]. Available at: https://doi.org/10.1007/s10238-022-00917-x.
18. Beccaria, C.G. *et al.* (2018) “Galectin-3 deficiency drives lupus-like disease by promoting spontaneous germinal centers formation via IFN-γ,” *Nature Communications*, 9(1). Available at: https://doi.org/10.1038/s41467-018-04063-5.
19. Beghini, F. *et al.* (2021) “Integrating taxonomic, functional, and strain-level profiling of diverse microbial communities with bioBakery 3,” *eLife*, 10. Available at: https://doi.org/10.7554/elife.65088.
20. Bernstein, C.N. *et al.* (2010) “World Gastroenterology Organization practice Guidelines for the Diagnosis and Management of IBD in 2010,” *Inflammatory Bowel Diseases*, 16(1), pp. 112–124. Available at: https://doi.org/10.1002/ibd.21048.
21. Bibiloni, R. *et al.* (2006) “The bacteriology of biopsies differs between newly diagnosed, untreated, Crohn’s disease and ulcerative colitis patients,” *Journal of Medical Microbiology*, 55(8), pp. 1141–1149. Available at: https://doi.org/10.1099/jmm.0.46498-0.
22. Bisanz, J.E. (2018) “qiime2R: Importing QIIME2 artifacts and associated data into R sessions. Available at https://github.com/jbisanz/qiime2R.
23. Bjelland, I. et al. (2002) “The validity of the Hospital Anxiety and Depression Scale. An updated literature review,” *Journal of Psychosomatic Research,* 52(2), p. 69-77. Available at https://doi.org/10.1016/s0022-3999(01)00296-3.
24. Blackwell, J.M. *et al.* (2020) “Prevalence and duration of gastrointestinal symptoms before diagnosis of inflammatory bowel disease and Predictors of timely Specialist Review: A Population-Based Study,” *Journal of Crohn’s and Colitis*, 15(2), pp. 203–211. Available at: https://doi.org/10.1093/ecco-jcc/jjaa146.
25. Block, M. *et al.* (2016) “Immunohistochemical Studies on Galectin Expression in Colectomised Patients with Ulcerative Colitis,” *BioMed Research International*, 2016, pp. 1–10. Available at: https://doi.org/10.1155/2016/5989128.
26. Bolyen, E. *et al* (2019) “Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2,” *Nature Biotechnology*, 37:852–857. Available at: https://doi.org/10.1038/s41587-019-0209-9.
27. Bray, R.H. and Curtis, J.T. (1957) “An Ordination of the Upland Forest Communities of Southern Wisconsin,” *Ecological Monographs*, 27(4), pp. 325–349. Available at: https://doi.org/10.2307/1942268.
28. Brazowski, E. *et al.* (2009) “Galectin-3 expression in pouchitis in patients with ulcerative colitis who underwent ileal pouch-anal anastomosis (IPAA),” *Pathology Research and Practice*, 205(8), pp. 551–558. Available at: https://doi.org/10.1016/j.prp.2009.02.001.

29. Brinchmann, M.F., Patel, D.M. and Iversen, M.J. (2018) “The Role of Galectins as Modulators of Metabolism and Inflammation,” *Mediators of Inflammation*, 2018, pp. 1–11. Available at: https://doi.org/10.1155/2018/9186940.
30. Byrd, K.M. and Gulati, A.S. (2021) “The ‘Gum–Gut’ Axis in Inflammatory Bowel Diseases: A Hypothesis-Driven Review of Associations and Advances,” *Frontiers in Immunology*, 12. Available at: https://doi.org/10.3389/fimmu.2021.620124.
31. Callahan, B.J. (2016) “DADA2: High-resolution sample inference from Illumina amplicon data,” *Nature Methods,* 13(7):581-3. Available at: https://doi:10.1038/nmeth.3869.
32. Cano, L.Q. *et al.* (2019) “Intracellular galectin-9 controls dendritic cell function by maintaining plasma membrane rigidity,” SSRN Electronic Journal [Preprint]. Available at: https://doi.org/10.2139/ssrn.3397114.
33. Caporaso, J.G. *et al*. (2012) “Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms,” *Multidisciplinary Journal of Microbial Ecology,* 6, p. 1621-1624. Available at: https://doi.org/10.1038/ismej.2012.8
34. Caporaso, J.G. *et al.* (2018) “EMP 16S Illumina Amplicon Protocol v1,” *Protocols.io*. Available at: https://doi.org/10.17504/protocols.io.nuudeww.
35. Cerrillo, E. *et al.* (2015) “Fecal calprotectin in ileal Crohn’s Disease,” *Inflammatory Bowel Diseases*, 21(7), pp. 1572–1579. Available at: https://doi.org/10.1097/mib.0000000000000404.
36. Chao, A. (1984) “Non-parametric estimation of the number of classes in a population,” *Scandinavian Journal of Statistics,* 11(4), p. 265-270. Available at: https://doi.org/10.2307/4615964.
37. Chen, H.-Y. *et al.* (2020) “Intracellular galectin-9 enhances proximal TCR signaling and potentiates autoimmune diseases,” *Journal of Immunology*, 204(5), pp. 1158–1172. https://doi.org/10.4049/jimmunol.1901114.
38. Cibor, D. *et al.* (2019) “Serum galectin 3, galectin 9 and galectin 3-binding proteins in patients with active and inactive inflammatory bowel disease.,” *Journal of Physiology and Pharmacology*, 70(1). Available at: https://doi.org/10.26402/jpp.2019.1.06.
39. Conte, M.R. *et al.* (2006) “Gut-associated bacterial microbiota in paediatric patients with inflammatory bowel disease,” *Gut*, 55(12), pp. 1760–1767. Available at: https://doi.org/10.1136/gut.2005.078824.
40. Cordero, R.Y. *et al.* (2022) “*Trans*-ancestry, Bayesian meta-analysis discovers 20 novel risk loci for inflammatory bowel disease in an African American, East Asian and European cohort,” *Human Molecular Genetics*, 32(5), pp. 873–882. Available at: https://doi.org/10.1093/hmg/ddac269.
41. Crohn’s & Colitis UK. (2023) “New research shows over 1 in 123 people in the UK living with Crohn’s or Colitis,” Available at: https://crohnsandcolitis.org.uk/news-stories/news-items/new-research-shows-over-1-in-123-people-in-uk-living-with-crohn-s-or-colitis (last accessed 26/03/2023).
42. Crohn’s and Colitis UK. (2023) “Cut the Crap,” Available at: https://crohnsandcolitis.org.uk/our-work/campaigns/cut-the-crap (Last accessed 14/07/2023)

43. Cummings, R.D. (2009) “The repertoire of glycan determinants in the human glycome,” Molecular BioSystems, 5(10), p. 1087. Available at: https://doi.org/10.1039/b907931a.
44. Czeisler, M.É. *et al.* (2020) “Delay or avoidance of medical care because of COVID-19–Related concerns — United States, June 2020,” *Morbidity and Mortality Weekly Report*, 69(36), pp. 1250–1257. Available at: https://doi.org/10.15585/mmwr.mm6936a4.
45. D’Amico, F. *et al.* (2020) “A practical guide for faecal calprotectin measurement: myths and realities,” *Journal of Crohn’s and Colitis*, 15(1), pp. 152–161. Available at: https://doi.org/10.1093/ecco-jcc/jjaa093.
46. D'Argenio, V. *et al.* (2013) “An altered gut microbiome profile in a child affected by Crohn's disease normalized after nutritional therapy,” American Journal of Gastroenterology, 108(5):851-2. Available at: https://doi:10.1038/ajg.2013.46.
47. Daperno, M. *et al.* (2004) “Development and validation of a new, simplified endoscopic activity score for Crohn’s disease: the SES-CD,” *Gastrointestinal Endoscopy*, 60(4), pp. 505–512. Available at: https://doi.org/10.1016/s0016-5107(04)01878-4.
48. Dardalhon, V. *et al.* (2010) “Tim-3/Galectin-9 Pathway: Regulation of Th1 Immunity through Promotion of CD11b+Ly-6G+ Myeloid Cells,” *Journal of Immunology*, 185(3), pp. 1383–1392. Available at: https://doi.org/10.4049/jimmunol.0903275.
49. De Lange, K.M. *et al.* (2017) “Genome-wide association study implicates immune activation of multiple integrin genes in inflammatory bowel disease,” *Nature Genetics*, 49(2), pp. 256–261. Available at: https://doi.org/10.1038/ng.3760.
50. De Meij, T.G.J. *et al.* (2018) “Variability of core microbiota in newly diagnosed treatment-naïve paediatric inflammatory bowel disease patients,” *PLOS ONE*, 13(8), p. e0197649. Available at: https://doi.org/10.1371/journal.pone.0197649.
51. DeRoche, T.C., Xiao, S.-Y. and Liu, X. (2014) “Histological evaluation in ulcerative colitis,” *Gastroenterology Report*, 2(3), pp. 178–192. Available at: https://doi.org/10.1093/gastro/gou031.
52. Diederen, K. *et al.* (2020) “Exclusive enteral nutrition mediates gut microbial and metabolic changes that are associated with remission in children with Crohn's disease,” *Nature Scientific Reports,*10(1):18879. Available at: https://doi:10.1038/s41598-020-75306-z.
53. Douglas, G.M. et al. (2018) “Multi-omics differentially classify disease state and treatment outcome in pediatric Crohn's disease,” *Microbiome*, 6(13). Available at: https://doi.org/10.1186/s40168-018-0398-3.
54. Douglas, G.M. *et al.* (2020) “PICRUSt2 for prediction of metagenome functions,” *Nature Biotechnology*, 38(6), pp. 685–688. Available at: https://doi.org/10.1038/s41587-020-0548-6.
55. Dhaliwal, A. *et al.* (2015) “Utility of faecal calprotectin in inflammatory bowel disease (IBD): what cut-offs should we apply?,” *Frontline Gastroenterology*, 6(1), pp. 14–19. Available at: https://doi.org/10.1136/flgastro-2013-100420.
56. DNA Genotek. (2023) “Storage Recommendations for samples collected using OMNIgene products from DNA Genotek,” Available at: https://dnagenotek.com/ROW/pdf/PD-PR-01036.pdf (last accessed 14/03/2023).

57. Dubnau, D. et al. (1965) “Gene conservation in Bacillus species. I. Conserved genetic and nucleic acid base sequence homologies,” Proceedings of the National Academy of Sciences, 54(2), pp. 491–498. Available at: https://doi.org/10.1073/pnas.54.2.491.
58. Durazzi, F. *et al.* (2021) “Comparison between 16S rRNA and shotgun sequencing data for the taxonomic characterization of the gut microbiota,” *Scientific Reports*, 11(1). Available at: https://doi.org/10.1038/s41598-021-82726-y.
59. Eccles, J.A. *et al.* (2020) “Inflammatory bowel disease psychological support pilot reduces inflammatory bowel disease symptoms and improves psychological wellbeing,” *Frontline Gastroenterology*, 12(2), pp. 154–157. Available at: https://doi.org/10.1136/flgastro-2019-101323.
60. El Mouzan, M. *et al.* (2017) “Fungal Microbiota Profile in Newly Diagnosed Treatment-naïve Children with Crohn’s Disease,” *Journal of Crohn S and Colitis*, 11(5), pp. 586–592. Available at: https://doi.org/10.1093/ecco-jcc/jjw197.
61. El Mouzan M. *et al.* (2018) “Microbiota profile in new-onset pediatric Crohn's disease: data from a non-Western population,” Gut Pathogens, 29;10:49. Available at: https://doi:10.1186/s13099-018-0276-3.
62. El Mouzan, M. *et al.* (2021) “Bacterial dysbiosis predicts the diagnosis of Crohn’s disease in Saudi children,” *Saudi Journal of Gastroenterology*, 27(3), p. 144. Available at: https://doi.org/10.4103/sjg.sjg_409_20.
63. Ellul, S. et al. (2021) “P673 Microbial diversity in newly diagnosed treatment naïve Inflammatory Bowel Disease patients,” Journal of Crohn S and Colitis, 15(Supplement_1), pp. S594–S595. Available at: https://doi.org/10.1093/ecco-jcc/jjab076.793.
64. Elmaghrawy, K. *et al.* (2022) “The Oral Microbiome in Treatment-Naïve Paediatric IBD Patients Exhibits Dysbiosis Related to Disease Severity that Resolves Following Therapy,” *Journal of Crohn S and Colitis*, 17(4), pp. 553–564. Available at: https://doi.org/10.1093/ecco-jcc/jjac155.
65. Engel, K.L. *et al.* (2021) “Newly Diagnosed Patients with Inflammatory Bowel Disease: The Relationship Between Perceived Psychological Support, Health-Related Quality of Life, and Disease Activity,” *Health Equity*, 5(1), pp. 42–48. Available at: https://doi.org/10.1089/heq.2020.0053.
66. Engevik, M.A. *et al.* (2020) “20 ELUCIDATING THE ROLE OF FUSOBACTERIUM NUCLEATUM IN INTESTINAL INFLAMMATION,” *Inflammatory Bowel Diseases*, 26(Supplement_1), p. S29. Available at: https://doi.org/10.1093/ibd/zaa010.070.
67. Fairbrass, K.M., Gracie, D.J. and Ford, A.C. (2022) “Relative contribution of disease activity and psychological health to prognosis of inflammatory bowel disease during 6.5 years of longitudinal Follow-Up,” *Gastroenterology*, 163(1), pp. 190-203.e5. Available at: https://doi.org/10.1053/j.gastro.2022.03.014.
68. Faith, D.P. (1992) “Conservation evaluation and phylogenetic diversity,” *Biological Conservation*, 61(1), pp. 1–10. Available at: https://doi.org/10.1016/0006-3207(92)91201-3.
69. Falony, G. et al. (2016) “Population-level analysis of gut microbiome variation,” *Science*, 352(6285), pp. 560–564. Available at: https://doi.org/10.1126/science.aad3503.

70. Falvey, J.D. et al. (2015) “Disease Activity Assessment in IBD,” Inflammatory Bowel Diseases, 21(4), pp. 824–831. Available at: https://doi.org/10.1097/mib.0000000000000341.
71. Fletcher, J., Cooper, S.C. and Swift, A. (2021) “Patient-Reported Outcomes in Inflammatory Bowel Disease: A Measurement of Effect in research and Clinical care,” *Gastroenterology Insights*, 12(2), pp. 225–237. Available at: https://doi.org/10.3390/gastroent12020020.
72. Fossum Moen, A.E. *et al.* (2018) “The prevalence and transcriptional activity of the mucosal microbiota of ulcerative colitis patients,” *Scientific Reports*, 8(1). Available at: https://doi.org/10.1038/s41598-018-35243-4.
73. Fox, J. and Weisberg, S. (2020). “*car: Companion to Applied Regression*.” [R package]. Retrieved from https://cran.r-project.org/package=car (last accessed 23/10/2024).
74. Franzosa, E.A. *et al.* (2018) “Gut microbiome structure and metabolic activity in inflammatory bowel disease,” *Nature Microbiology*, 4(2), pp. 293–305. Available at: https://doi.org/10.1038/s41564-018-0306-4.
75. Frau, A. *et al.* (2021) “Inter-kingdom relationships in Crohn’s disease explored using a multi-omics approach,” *Gut Microbes*, 13(1). Available at: https://doi.org/10.1080/19490976.2021.1930871.
76. Friesen L, Kroc E, Zumbo, B. “*Psychometrics & Post-Data Analysis: Test ROC*,” 2019. [jamovi module]. Retrieved from https://github.com/lucasjfriesen/jamoviPsychoPDA. (last accessed 04/01/2024).
77. Fritsch, J. and Abreu, M.T. (2019) “The microbiota and the immune response: What is the chicken and what is the egg?,” *Gastrointestinal Endoscopy Clinics of North America*, 29(3), pp. 381–393. Available at: https://doi.org/10.1016/j.giec.2019.02.005.
78. Fro’lova, L. *et al.* (2009) “Detection of galectin-3 in patients with inflammatory bowel diseases: new serum marker of active forms of IBD?,” *Inflammation Research*, 58(8), pp. 503–512. Available at: https://doi.org/10.1007/s00011-009-0016-8.
79. Funasaka, T., Raz, A. and Nangia-Makker, P. (2014) “Galectin-3 in angiogenesis and metastasis,” *Glycobiology*, 24(10), pp. 886–891. https://doi.org/10.1093/glycob/cwu086.
80. Galipeau, H.J. *et al.* (2021) “Novel fecal biomarkers that precede clinical diagnosis of ulcerative colitis,” *Gastroenterology*, 160(5), pp. 1532–1545. Available at https://doi.org/10.1053/j.gastro.2020.12.004.
81. Geremia, A. *et al.* (2011) “IL-23–responsive innate lymphoid cells are increased in inflammatory bowel disease,” *Journal of Experimental Medicine*, 208(6), pp. 1127–1133. Available at: https://doi.org/10.1084/jem.20101712.
82. Ge, X.N. *et al.* (2010) “Allergen-Induced airway remodeling is impaired in Galectin-3–Deficient mice,” *Journal of Immunology*, 185(2), pp. 1205–1214. Available at: https://doi.org/10.4049/jimmunol.1000039.
83. Gevers, D. *et al.* (2014) “The Treatment-Naive Microbiome in New-Onset Crohn’s Disease,” *Cell Host & Microbe*, 15(3), pp. 382–392. Available at: https://doi.org/10.1016/j.chom.2014.02.005.
84. Ghosh, S. *et al.* (2017) “Development of the IBD disk,” *Inflammatory Bowel Diseases*, 23(3), pp. 333–340. Available at: https://doi.org/10.1097/mib.0000000000001033.

85. Giovannone, N. *et al.* (2018) “Galectin-9 suppresses B cell receptor signaling and is regulated by I-branching of N-glycans,” *Nature Communications*, 9(1). Available at: https://doi.org/10.1038/s41467-018-05770-9.
86. Gisbert, J.P. et al. (2009) “Fecal calprotectin and lactoferrin for the prediction of inflammatory bowel disease relapse,” *Inflammatory Bowel Diseases*, 15(8), pp. 1190–1198. Available at: https://doi.org/10.1002/ibd.20933.
87. Gomez, A. *et al.* (2019) “Plasticity in the human gut microbiome defies evolutionary constraints,” *mSphere*, 4(4). Available at: https://doi.org/10.1128/msphere.00271-19.
88. Gophna, U. *et al.* (2006) “Differences between Tissue-Associated Intestinal Microfloras of Patients with Crohn’s Disease and Ulcerative Colitis,” *Journal of Clinical Microbiology*, 44(11), pp. 4136–4141. Available at: https://doi.org/10.1128/jcm.01004-06.
89. Gophna, U., Konikoff, T. and Nielsen, H. (2017) “*Oscillospira* and related bacteria - From metagenomic species to metabolic features,” *Environmental Microbiology*, 19(3), pp. 835–841. Available at: https://doi.org/10.1111/1462-2920.13658.
90. Gorbach, S.L. *et al.* (1968) “Studies of intestinal microflora,” *Gastroenterology*, 54(4), pp. 575–587. Available at: https://doi.org/10.1016/s0016-5085(68)80072-1.
91. Gordón-Alonso, M., Bruger, A.M. and Van Der Bruggen, P. (2018) “Extracellular galectins as controllers of cytokines in hematological cancer,” *Blood*, 132(5), pp. 484–491. Available at: https://doi.org/10.1182/blood-2018-04-846014.
92. Graff, L.A., Walker, J.R. and Bernstein, C.N. (2009) “Depression and anxiety in inflammatory bowel disease: A review of comorbidity and management,” *Inflammatory Bowel Diseases*, 15(7), pp. 1105–1118. Available at: https://doi.org/10.1002/ibd.20873.
93. Graham, D.J. and Xavier, R.J. (2020) “Pathway paradigms revealed from the genetics of inflammatory bowel disease,” *Nature*, 578(7796), pp. 527–539. Available at: https://doi.org/10.1038/s41586-020-2025-2.
94. Grover, Z. *et al.* (2016) “633 The Relative Abundances of Dorea and Faecalibacterium spp. in the Mucosa Associated Microbiome of Newly Diagnosed Children With Crohn’s Disease Are Differentially Affected by Exclusive Enteral Nutrition,” *Gastroenterology*, 150(4), pp. S132–S133. Available at: https://doi.org/10.1016/s0016-5085(16)30547-9.
95. Guo, D. *et al* (2022) “Exploring Different Effects of Exclusive Enteral Nutrition (EEN) and Corticosteroids on the Gut Microbiome in Crohn's Disease Based on a Three-Stage Strategy,” *Gastroenterology Research and Practice,* 27;2022:6147124. Available at: https://doi:10.1155/2022/6147124.
96. Haifer, C. *et al.* (2022) “Lyophilised oral faecal microbiota transplantation for ulcerative colitis (LOTUS): a randomised, double-blind, placebo-controlled trial,” T*he Lancet. Gastroenterology & Hepatology*, 7(2), pp. 141–151. Available at: https://doi.org/10.1016/s2468-1253(21)00400-3.
97. Halfvarson, J. *et al.* (2017) “Dynamics of the human gut microbiome in inflammatory bowel disease,” *Nature Microbiology*, 2(5). Available at: https://doi.org/10.1038/nmicrobiol.2017.4.
98. Hansen, R.A. *et al.* (2012) “Microbiota of De-Novo Pediatric IBD: Increased Faecalibacterium Prausnitzii and Reduced Bacterial Diversity in Crohn’s But

Not in Ulcerative Colitis," *The American Journal of Gastroenterology*, 107(12), pp. 1913–1922. Available at: https://doi.org/10.1038/ajg.2012.335.
99. Hansen, R.A. *et al.* (2013) "The microaerophilic microbiota of De-Novo paediatric inflammatory bowel Disease: the BISCUIT Study," *PLOS ONE*, 8(3), p. e58825. Available at: https://doi.org/10.1371/journal.pone.0058825.
100. Hart, A. *et al.* (2005) "Characteristics of Intestinal Dendritic Cells in Inflammatory Bowel Diseases," *Gastroenterology*, 129(1), pp. 50–65. Available at: https://doi.org/10.1053/j.gastro.2005.05.013.
101. Hart, G.W. (2023) "Overview of glycobiology," in *Elsevier eBooks*, pp. 297–308. Available at: https://doi.org/10.1016/b978-0-12-821618-7.00233-9.
102. Hart, L. et al. (2019) "Faecal calprotectin predicts endoscopic and histological activity in clinically quiescent ulcerative colitis," *Journal of Crohns and Colitis*, 14(1), pp. 46–52. Available at: https://doi.org/10.1093/ecco-jcc/jjz107.
103. Hart, L. *et al.* (2020) "Effect of Exclusive Enteral Nutrition and Corticosteroid Induction Therapy on the Gut Microbiota of Pediatric Patients with Inflammatory Bowel Disease," *Nutrients*, 12(6), p. 1691. Available at: https://doi.org/10.3390/nu12061691.
104. Hartley, M.G. *et al*. (1992) "The rectal mucosa-associated microflora in patients with ulcerative colitis," *Journal of Medical Microbiology*, 36(2):96-103. Available at: https://doi:10.1099/00222615-36-2-96.
105. Hartley, M.G. *et al*. (1993) "Adhesive and hydrophobic properties of Escherichia coli from the rectal mucosa of patients with ulcerative colitis," *Gut,* 34(1):63-7. Available at: https://doi:10.1136/gut.34.1.63.
106. Hartley, M.G. *et al*. (1996) "Sulphasalazine treatment and the colorectal mucosa-associated flora in ulcerative colitis," *Alimentary Pharmacology & Therapeutics*, 10(2):157-63. Available at: https://doi:10.1046/j.1365-2036.1996.723115000.
107. Harvey, R.W. and Bradshaw, J.D. (1980) "A SIMPLE INDEX OF CROHN'S-DISEASE ACTIVITY," *The Lancet*, 315(8167), p. 514. Available at: https://doi.org/10.1016/s0140-6736(80)92767-1.
108. Hicks, A., Hamlin, P.J. and Selinger, C. (2020) "The Association of Introducing a Faecal Calprotectin Testing Pathway for Suspected Inflammatory Bowel Disease in Primary Care and Time to Diagnosis or Treatment," *Inflammatory Intestinal Diseases*, 5(4), pp. 191–199. Available at: https://doi.org/10.1159/000509907.
109. Higgins, J. et al. (2019) "*Cochrane Handbook for Systematic Reviews of Interventions*. 2nd Edition." Chichester (UK): John Wiley & Sons.
110. Hillmann, B. *et al.* (2018) "Evaluating the information content of shallow shotgun metagenomics," *MSystems*, 3(6). Available at: https://doi.org/10.1128/msystems.00069-18.
111. Hiippala, K. *et al.* (2020) "Isolation of Anti-Inflammatory and Epithelium Reinforcing Bacteroides and Parabacteroides Spp. from A Healthy Fecal Donor," *Nutrients*, 12(4), p. 935. Available at: https://doi.org/10.3390/nu12040935.
112. Hinnant, L. *et al.* (2024) "Consensus Statement on Managing Anxiety and Depression in Individuals with Inflammatory Bowel Disease," *Inflammatory Bowel Diseases* [Preprint]. Available at: https://doi.org/10.1093/ibd/izae151.
113. Hollister, E.B. et al. (2015) "Structure and function of the healthy pre-adolescent pediatric gut microbiome," *Microbiome*, 3(1). Available at: https://doi.org/10.1186/s40168-015-0101-x.

114. Holm, S. (1979) “A Simple Sequentially Rejective Multiple Test Procedure,” *Scandinavian Journal of Statistics*, 6, pp. 65–70. Available at: https://doi.org/10.2307/4615733.
115. Huttenhower, C. *et al.* (2012) “Structure, function and diversity of the healthy human microbiome,” *Nature*, 486(7402), pp. 207–214. Available at: https://doi.org/10.1038/nature11234.
116. Hyams, J.S. *et al.* (2019) “Clinical and biological predictors of response to standardised paediatric colitis therapy (PROTECT): a multicentre inception cohort study,” *The Lancet*, 393(10182), pp. 1708–1720. Available at: https://doi.org/10.1016/s0140-6736(18)32592-3.
117. IBD UK. (2021) “Crohn’s and colitis care in the UK: The hidden cost and a vision for change,” Available at: https://s3.eu-west-2.amazonaws.com/files.ibduk.org/documents/CROJ8096-IBD-National-Report-WEB-210427-2.pdf (Last accessed 14/07/2023)
118. IBD UK. (2023) “IBD Standards,” Available at: https://www.ibduk.org/ibd-standards (last accessed 06/03/2023).
119. IBD UK. (2024) “2024 IBD UK Report: The State of IBD Care in the UK,” Available at: https://ibduk.org/reports/2024-ibd-uk-report-the-state-of-ibd-care-in-the-uk (last accessed 22/04/2025)
120. Iebba, V. *et al.* (2013) “Higher Prevalence and Abundance of Bdellovibrio bacteriovorus in the Human Gut of Healthy Subjects,” *PLoS ONE*, 8(4), p. e61608. Available at: https://doi.org/10.1371/journal.pone.0061608.
121. Ilarregui, J.M. et al. (2009) “Tolerogenic signals delivered by dendritic cells to T cells through a galectin-1-driven immunoregulatory circuit involving interleukin 27 and interleukin 10,” Nature Immunology, 10(9), pp. 981–991. Available at: https://doi.org/10.1038/ni.1772.
122. Imam, T. *et al.* (2018) “Effector T Helper Cell Subsets in Inflammatory Bowel Diseases,” *Frontiers in Immunology*, 9. Available at: https://doi.org/10.3389/fimmu.2018.01212.
123. Iqbal, A. *et al.* (2021) “Galectin-9 mediates neutrophil capture and adhesion in a CD44 and β2 integrin-dependent manner,” *The FASEB Journal*, 36(1). Available at: https://doi.org/10.1096/fj.202100832r.
124. Iwatani, S. *et al.* (2020) “Oligosaccharide-dependent anti-inflammatory role of galectin-1 for macrophages in ulcerative colitis,” *Journal of Gastroenterology and Hepatology*, 35(12), pp. 2158–2169. Available at: https://doi.org/10.1111/jgh.15097.
125. Jaccard, P. (1912) “THE DISTRIBUTION OF THE FLORA IN THE ALPINE ZONE.1,” *New Phytologist*, 11(2), pp. 37–50. Available at: https://doi.org/10.1111/j.1469-8137.1912.tb05611.x.
126. JASP Team. (2024). “JASP (Version 0.18.3)” [Computer software]. Available at: https://jasp-stats.org/download/
127. Jayaraman, P. *et al.* (2010) “Tim3 binding to galectin-9 stimulates antimicrobial immunity,” *Journal of Experimental Medicine*, 207(11), pp. 2343–2354. Available at: https://doi.org/10.1084/jem.20100687.
128. Jayasooriya, N. et al. (2023) “Systematic review with meta-analysis: Time to diagnosis and the impact of delayed diagnosis on clinical outcomes in inflammatory bowel disease,” *Alimentary Pharmacology & Therapeutics*, 57(6), pp. 635–652. Available at: https://doi.org/10.1111/apt.17370.
129. Jensen-Jarolim, E. *et al.* (2001) “Anti-Galectin-3 IgG autoantibodies in patients with Crohn’s disease characterized by means of phage display

peptide libraries," *Journal of Clinical Immunology*, 21(5), pp. 348–356. Available at: https://doi.org/10.1023/a:1012240719801.
130. Jensen-Jarolim, E. *et al.* (2002) "The constitutive expression of galectin-3 is downregulated in the intestinal epithelia of Crohn's disease patients, and tumour necrosis factor alpha decreases the level of galectin-3-specific mRNA in HCT-8 cells," *European Journal of Gastroenterology & Hepatology*, 14(2), pp. 145–152. Available at: https://doi.org/10.1097/00042737-200202000-00008.
131. Johannes, L., Jacob, R. and Leffler, H. (2018) "Galectins at a glance," *Journal of Cell Science*, 131(9). Available at: https://doi.org/10.1242/jcs.208884.
132. Jones, G.T. *et al.* (2018) "Faecal calprotectin and Magnetic resonance enterography in Ileal Crohn's Disease: Correlations between disease activity and Long-Term Follow-Up," *Journal of Crohn's and Colitis*, 13(4), pp. 442–450. Available at: https://doi.org/10.1093/ecco-jcc/jjy187.
133. Jostins, L. *et al.* (2012) "Host–microbe interactions have shaped the genetic architecture of inflammatory bowel disease," *Nature*, 491(7422), pp. 119–124. Available at: https://doi.org/10.1038/nature11582.
134. Jovanovic, M. *et al.* (2019) "Metabolic syndrome attenuates ulcerative colitis: Correlation with interleukin-10 and galectin-3 expression," *World Journal of Gastroenterology*, 25(43), pp. 6465–6482. Available at: https://doi.org/10.3748/wjg.v25.i43.6465.
135. Jun, X. *et al.* (2019) "Alteration of fungal microbiota after 5-ASA treatment in UC patients," *Inflammatory Bowel Diseases*, 26(3), pp. 380–390. Available at: https://doi.org/10.1093/ibd/izz207.
136. Juyal, G. *et al.* (2021) "Correlation between faecal microbial taxa and ulcerative colitis in different phases of disease activity in a north Indian cohort," medRxiv. Available at: https://doi:10.1101/2021.12.12.21267614.
137. Kaakoush, N.O. *et al.* (2012) "Microbial Dysbiosis in Pediatric Patients with Crohn's Disease," *Journal of Clinical Microbiology*, 50(10), pp. 3258–3266. Available at: https://doi.org/10.1128/jcm.01396-12.
138. Kageyama, S. *et al.* (2023) "High-Resolution detection of translocation of oral bacteria to the gut," *Journal of Dental Research*, 102(7), pp. 752–758. Available at: https://doi.org/10.1177/00220345231160747.
139. Kansal, S. *et al.* (2019) "Variation of Gut Mucosal Microbiome With Anti-Saccharomyces cerevisiae Antibody Status in Pediatric Crohn Disease," *Journal of Pediatric Gastroenterology and Nutrition*, 69(6), pp. 696–703. Available at: https://doi.org/10.1097/mpg.0000000000002461.
140. Kashio, Y. et al. (2003) "Galectin-9 induces apoptosis through the Calcium-Calpain-Caspase-1 pathway," The Journal of Immunology, 170(7), pp. 3631–3636. Available at: https://doi.org/10.4049/jimmunol.170.7.3631.
141. Katsaros, M. *et al.* (2023) "P370 Correlation of the IBD Disk with intestinal ultrasound in patients with inflammatory bowel disease," *Journal of Crohn's and Colitis*, 17(Supplement_1), pp. i499–i500. Available at: https://doi.org/10.1093/ecco-jcc/jjac190.0500.
142. Kellermayer, R. *et al.* (2012) "Microbiota separation and C-reactive protein elevation in treatment-naïve pediatric granulomatous Crohn disease," *Journal of Pediatric Gastroenterology and Nutrition*, 55(3), pp. 243–250. Available at: https://doi.org/10.1097/mpg.0b013e3182617c16.

143. Kelley, N. *et al.* (2019) “The NLRP3 Inflammasome: An Overview of mechanisms of activation and regulation,” *International Journal of Molecular Sciences*, 20(13), p. 3328. Available at: https://doi.org/10.3390/ijms20133328.
144. Kessel, C. *et al.* (2021) “Serum biomarkers confirming stable remission in inflammatory bowel disease,” *Scientific Reports*, 11(1). Available at: https://doi.org/10.1038/s41598-021-86251-w.
145. Kevans, D. *et al.* (2015) “Characterization of Intestinal Microbiota in Ulcerative Colitis Patients with and without Primary Sclerosing Cholangitis,” *Journal of Crohn S and Colitis*, 10(3), pp. 330–337. Available at: https://doi.org/10.1093/ecco-jcc/jjv204.
146. Khan, I.A. *et al* (2021) “Differential prevalence of pathobionts and host gene polymorphisms in chronic inflammatory intestinal diseases: Crohn's disease and intestinal tuberculosis,” *PLoS One*, 16(8):e0256098. Available at: https://doi:10.1371/journal.pone.0256098.
147. Kim, B.Y. *et al.* (2017) “Deciphering Diversity Indices for a Better Understanding of Microbial Communities,” *Journal of Microbiology and Biotechnology*, 27(12), pp. 2089–2093. Available at: https://doi.org/10.4014/jmb.1709.09027.
148. King, D. *et al.* (2020) “Changing patterns in the epidemiology and outcomes of inflammatory bowel disease in the United Kingdom: 2000-2018,” *Alimentary Pharmacology and Therapeutics*, 51(10), pp. 922–934. Available at: https://doi.org/10.1111/apt.15701.
149. Kirsner, J.B. and Palmer, W.L. (1954) “Ulcerative colitis,” *Journal of the American Medical Association*, 155(4), p. 341. Available at: https://doi.org/10.1001/jama.155.4.341.
150. Kmieć, Z., Cyman, M. and Ślebioda, T.J. (2017) “Cells of the innate and adaptive immunity and their interactions in inflammatory bowel disease,” *Advances in Medical Sciences*, 62(1), pp. 1–16. Available at: https://doi.org/10.1016/j.advms.2016.09.001.
151. Kok, K. *et al.* (2022) “Understanding and managing psychological disorders in patients with inflammatory bowel disease: a practical guide,” *Frontline Gastroenterology*, 14(1), pp. 78–86. Available at: https://doi.org/10.1136/flgastro-2022-102094.
152. Kolde, R. (2019) “pheatmap: Pretty Heatmaps,” R package version 1.0.12. Available at: https://CRAN.R-project.org/package=pheatmap.
153. Kong, L. *et al.* (2023) “The landscape of immune dysregulation in Crohn’s disease revealed through single-cell transcriptomic profiling in the ileum and colon,” *Immunity*, 56(2), pp. 444-458.e5. Available at: https://doi.org/10.1016/j.immuni.2023.01.002.
154. Kostic, A., Xavier, R.J. and Gevers, D. (2014) “The microbiome in Inflammatory Bowel Disease: current status and the future ahead,” *Gastroenterology*, 146(6), pp. 1489–1499. Available at: https://doi.org/10.1053/j.gastro.2014.02.009.
155. Kowalska-Duplaga, K. et al (2019) “Differences in the intestinal microbiome of healthy children and patients with newly diagnosed Crohn's disease”. *Nature Scientific Reports,* 9(1):18880. Available at https://doi:10.1038/s41598-019-55290-9.
156. Kugathasan, S. *et al.* (2017) “Prediction of complicated disease course for children newly diagnosed with Crohn’s disease: a multicentre inception cohort

study," *The Lancet*, 389(10080), pp. 1710–1718. Available at: https://doi.org/10.1016/s0140-6736(17)30317-3.
157. Kverka, M. *et al.* (2010) "Oral administration of Parabacteroides distasonis antigens attenuates experimental murine colitis through modulation of immunity and microbiota composition," *Clinical & Experimental Immunology*, 163(2), pp. 250–259. Available at: https://doi.org/10.1111/j.1365-2249.2010.04286.x.
158. Kyle, B.D. *et al.* (2020) "Fecal calprotectin, CRP and leucocytes in IBD patients: Comparison of biomarkers with biopsy results," *Journal of the Canadian Association of Gastroenterology*, 4(2), pp. 84–90. Available at: https://doi.org/10.1093/jcag/gwaa009.
159. Lamb, C.A. *et al.* (2019) "British Society of Gastroenterology consensus guidelines on the management of inflammatory bowel disease in adults," *Gut*, 68(Suppl 3), pp. s1–s106. Available at: https://doi.org/10.1136/gutjnl-2019-318484. Erratum in: (2021) Gut, 70(4), p. 1. Available at: https://doi.org/10.1136/gutjnl-2019-318484corr1.
160. Langmead, B. and Salzberg, S.L. (2012) "Fast gapped-read alignment with Bowtie 2," Nature Methods, 9(4), pp. 357–359. Available at: https://doi.org/10.1038/nmeth.1923.
161. Laudadio, I. *et al.* (2018) "Quantitative Assessment of Shotgun Metagenomics and 16S rDNA Amplicon Sequencing in the Study of Human Gut Microbiome," *Omics a Journal of Integrative Biology*, 22(4), pp. 248–254. Available at: https://doi.org/10.1089/omi.2018.0013.
162. Larsen, J.M. *et al.* (2012) "Divergent Pro-Inflammatory Profile of Human Dendritic Cells in Response to Commensal and Pathogenic Bacteria Associated with the Airway Microbiota," *PLOS ONE*, 7(2), p. e31976. Available at: https://doi.org/10.1371/journal.pone.0031976.
163. Le Berre, C. *et al.* (2020) "VALIDation of the IBD-DISK instrument for assessing disability in inflammatory bowel diseases in a French cohort: the VALIDate Study," *Journal of Crohn's and Colitis*, 14(11), pp. 1512–1523. Available at: https://doi.org/10.1093/ecco-jcc/jjaa100.
164. Lecky, D.M., Hawking, M.K. and McNulty, C.A. (2014) "Patients' perspectives on providing a stool sample to their GP: a qualitative study," *British Journal of General Practice*, 64(628), pp. e684–e693. Available at: https://doi.org/10.3399/bjgp14x682261.
165. Lee, E. *et al.* (2023) "Positive faecal immunochemical test predicts the onset of inflammatory bowel disease: A nationwide, propensity score-matched study," *Frontiers in Immunology*, 14. Available at: https://doi.org/10.3389/fimmu.2023.1128736.
166. Lederberg, J. and McCray, A.T. (2001) "`Ome Sweet `Omics--A Genealogical Treasury of Words," *The Scientist*, 15(7), p. 8. Available at: https://go.gale.com/ps/i.do?id=GALE%7CA73535513&v=2.1&it=r&linkaccess=abs&issn=08903670&p=AONE&sw=w&userGroupName=anon%7Ec3966c0c.
167. Lenth, R. (2020). "*emmeans: Estimated Marginal Means, aka Least-Squares Means*," [R package]. Available at: https://cran.r-project.org/package=emmeans.
168. Levine, A. *et al.* (2019) "Crohn's disease exclusion diet plus partial enteral nutrition induces sustained remission in a randomized controlled trial,"

Gastroenterology, 157(2), pp. 440-450.e8. Available at: https://doi.org/10.1053/j.gastro.2019.04.021.
169. Liao, C et al. (2024) “Oral bacteria relative abundance in faeces increases due to gut microbiota depletion and is linked with patient outcomes,” *Nat Microbiol* **9**, 1555–1565 (2024). Available at: https://doi.org/10.1038/s41564-024-01680-3.
170. Lightfoot, A., McGettrick, H.M. and Iqbal, A. (2021) “Vascular endothelial galectins in leukocyte trafficking,” *Frontiers in Immunology*, 12. Available at: https://doi.org/10.3389/fimmu.2021.687711.
171. Lin, C.-Y. *et al.* (2020) “Autophagy Receptor Tollip Facilitates Bacterial Autophagy by Recruiting Galectin-7 in Response to Group A Streptococcus Infection,” *Frontiers in Cellular and Infection Microbiology*, 10. Available at: https://doi.org/10.3389/fcimb.2020.583137.
172. Lippert, E. *et al.* (2008) “Regulation of galectin-3 function in mucosal fibroblasts: potential role in mucosal inflammation,” *Clinical and Experimental Immunology*, 152(2), pp. 285–297. Available at: https://doi.org/10.1111/j.1365-2249.2008.03618.x.
173. Liu, F.-T. and Rabinovich, G.A. (2010) “Galectins: regulators of acute and chronic inflammation,” *Annals of the New York Academy of Sciences*, 1183(1), pp. 158–182. Available at: https://doi.org/10.1111/j.1749-6632.2009.05131.x.
174. Liu, F.-T. and Stowell, S.R. (2023) “The role of galectins in immunity and infection,” Nature Reviews. Immunology, 23(8), pp. 479–494. Available at: https://doi.org/10.1038/s41577-022-00829-7.
175. Lloyd-Price, J. *et al.* (2019) “Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases,” *Nature*, 569(7758), pp. 655–662. Available at: https://doi.org/10.1038/s41586-019-1237-9.
176. Lo, T.-H. *et al.* (2021) “Galectin-3 promotes noncanonical inflammasome activation through intracellular binding to lipopolysaccharide glycans,” *Proceedings of the National Academy of Sciences of the United States of America*, 118(30). https://doi.org/10.1073/pnas.2026246118.
177. Love, M.I., Huber, W. and Anders, S. (2014) “Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2,” *Genome Biology*, 15(12). Available at: https://doi.org/10.1186/s13059-014-0550-8.
178. Lozupone, C.A. and Knight, R. (2005) “UniFrac: a New Phylogenetic Method for Comparing Microbial Communities,” *Applied and Environmental Microbiology*, 71(12), pp. 8228–8235. Available at: https://doi.org/10.1128/aem.71.12.8228-8235.2005.
179. Lv, Y. *et al.* (2023) “The impact of exclusive enteral nutrition on the gut microbiome and bile acid metabolism in pediatric Crohn’s disease,” *Clinical Nutrition*, 42(2), pp. 116–128. Available at: https://doi.org/10.1016/j.clnu.2022.11.018.
180. Maaser, C. *et al.* (2018) “ECCO-ESGAR Guideline for Diagnostic Assessment in IBD Part 1: Initial diagnosis, monitoring of known IBD, detection of complications,” *Journal of Crohn’s and Colitis*, 13(2), pp. 144-164K. Available at: https://doi.org/10.1093/ecco-jcc/jjy113.
181. Macklaim, J. et al. (2022) “OMNIgene GUT provides ambient temperature stabilization of pediatric fecal samples for Microbiome Sampling,” Available at: https://dnagenotek.com/ROW/pdf/PD-WP-00057.pdf (last accessed 14/03/2023).

182. Macpherson, S. *et al.* (2021) “P125 Faecal immunochemical test (FIT) in primary care as a rule out test for IBD,” *Gut*, 70, p. A107. Available at: https://doi.org/10.1136/gutjnl-2020-bsgcampus.200.
183. Magnusson, M.K. *et al.* (2016) “Anti-TNF Therapy Response in Patients with Ulcerative Colitis Is Associated with Colonic Antimicrobial Peptide Expression and Microbiota Composition,” *Journal of Crohn’s and Colitis*, 10(8), pp. 943–952. Available at: https://doi.org/10.1093/ecco-jcc/jjw051.
184. Magnusson, M.K. *et al.* (2017) “The Mucosal Antibacterial Response Profile and Fecal Microbiota Composition Are Linked to the Disease Course in Patients with Newly Diagnosed Ulcerative Colitis,” *Inflammatory Bowel Diseases*, 23(6), pp. 956–966. Available at: https://doi.org/10.1097/mib.0000000000001130.
185. Mallick, H. *et al.* (2021) “Multivariable association discovery in population-scale meta-omics studies,” *PLOS Computational Biology*, 17(11), p. e1009442. Available at: https://doi.org/10.1371/journal.pcbi.1009442.
186. Margulies, M. *et al.* (2005) “Genome sequencing in microfabricated high-density picolitre reactors,” *Nature*, 437(7057), pp. 376–380. Available at: https://doi.org/10.1038/nature03959.
187. Maringe, C. *et al.* (2020) “The impact of the COVID-19 pandemic on cancer deaths due to delays in diagnosis in England, UK: a national, population-based, modelling study,” *Lancet Oncology*, 21(8), pp. 1023–1034. Available at: https://doi.org/10.1016/s1470-2045(20)30388-0.
188. Markovic, B.S. *et al.* (2016) “Galectin-3 plays an important pro-inflammatory role in the induction phase of acute colitis by promoting activation of NLRP3 inflammasome and production of IL-1B in macrophages,” *Journal of Crohn’s and Colitis*, 10(5), pp. 593–606. Available at: https://doi.org/10.1093/ecco-jcc/jjw013.
189. Mars, R.A.T. *et al.* (2020) “Longitudinal multi-omics reveals Subset-Specific mechanisms underlying irritable bowel syndrome,” *Cell*, 182(6), pp. 1460-1473.e17. Available at: https://doi.org/10.1016/j.cell.2020.08.007.
190. Martinon, F. *et al.* (2004) “Identification of bacterial muramyl dipeptide as activator of the NALP3/Cryopyrin inflammasome,” *Current Biology*, 14(21), pp. 1929–1934. Available at: https://doi.org/10.1016/j.cub.2004.10.027.
191. Massazza, A. *et al.* (2022) “The association between uncertainty and mental health: a scoping review of the quantitative literature,” *Journal of Mental Health*, 32(2), pp. 480–491. Available at: https://doi.org/10.1080/09638237.2021.2022620.
192. McMurdie, P.J. and Holmes, S. (2013) “phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data,” *PLoS ONE*, 8(4), p. e61217. https://doi.org/10.1371/journal.pone.0061217.
193. McQueenie, R. et al. (2019) “Morbidity, mortality and missed appointments in healthcare: a national retrospective data linkage study,” BMC Medicine, 17(1). Available at: https://doi.org/10.1186/s12916-018-1234-0.
194. Mehta, R.S. *et al.* (2023) “Gut microbial metabolism of 5-ASA diminishes its clinical efficacy in inflammatory bowel disease,” *Nature Medicine*, 29(3), pp. 700–709. https://doi.org/10.1038/s41591-023-02217-7.
195. Miclotte, L. *et al.* (2020) “Dietary Emulsifiers Alter Composition and Activity of the Human Gut Microbiota in vitro, Irrespective of Chemical or Natural Emulsifier Origin,” *Frontiers in Microbiology*, 11. Available at: https://doi.org/10.3389/fmicb.2020.577474.

196. Mirzayi, C. *et al.* (2021) “Reporting guidelines for human microbiome research: the STORMS checklist,” *Nature Medicine*, 27(11), pp. 1885–1892. https://doi.org/10.1038/s41591-021-01552-x.
197. Moar, P. and Tandon, R. (2021) “Galectin-9 as a biomarker of disease severity,” *Cellular Immunology*, 361, p. 104287. Available at: https://doi.org/10.1016/j.cellimm.2021.104287.
198. Moeller, A.H. (2017c) “The shrinking human gut microbiome,” *Current Opinion in Microbiology*, 38, pp. 30–35. Available at: https://doi.org/10.1016/j.mib.2017.04.002.
199. Mokkink, L.B. *et al.* (2017) “COSMIN Risk of Bias checklist for systematic reviews of Patient-Reported Outcome Measures,” *Quality of Life Research*, 27(5), pp. 1171–1179. Available at: https://doi.org/10.1007/s11136-017-1765-4.
200. Morgan, X.C. *et al.* (2012) “Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment,” *GenomeBiology.com (London. Print)*, 13(9), p. R79. Available at: https://doi.org/10.1186/gb-2012-13-9-r79.
201. Morimoto, K. *et al.* (2011) “Dysregulated upregulation of T-cell immunoglobulin and mucin domain-3 on mucosal T helper 1 cells in patients with Crohn’s disease,” *Scandinavian Journal of Gastroenterology*, 46(6), pp. 701–709. Available at: https://doi.org/10.3109/00365521.2011.568518.
202. Mottawea, W. *et al.* (2016) “Altered intestinal microbiota–host mitochondria crosstalk in new onset Crohn’s disease,” *Nature Communications*, 7(1). Available at: https://doi.org/10.1038/ncomms13419.
203. Muegge, B.D. *et al.* (2011) “Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans,” *Science*, 332(6032), pp. 970–974. Available at: https://doi.org/10.1126/science.1198719.
204. Mukhopadhya, I. *et al.* (2015) “The fungal microbiota of de-novo paediatric inflammatory bowel disease,” *Microbes and Infection*, 17(4), pp. 304–310. Available at: https://doi.org/10.1016/j.micinf.2014.12.001.
205. Müller, S. *et al.* (2006) “Galectin-3 modulates T cell activity and is reduced in the inflamed intestinal epithelium in IBD,” *Inflammatory Bowel Diseases*, 12(7), pp. 588–597. Available at: https://doi.org/10.1097/01.mib.0000225341.37226.7c.
206. Mullish, B.H. *et al.* (2020) “The gut microbiome: what every gastroenterologist needs to know,” *Frontline Gastroenterology*, 12(2), pp. 118–127. Available at: https://doi.org/10.1136/flgastro-2019-101376.
207. Nabi, I.R., Shankar, J. and Dennis, J.W. (2015) “The galectin lattice at a glance,” *Journal of Cell Science*, 128(13), pp. 2213–2219. Available at: https://doi.org/10.1242/jcs.151159.
208. Nagata, N. *et al.* (2019) “Effects of bowel preparation on the human gut microbiome and metabolome,” *Scientific Reports*, 9(1). Available at: https://doi.org/10.1038/s41598-019-40182-9 .
209. Nava, P. *et al.* (2010) “Interferon-γ Regulates Intestinal Epithelial Homeostasis through Converging β-Catenin Signaling Pathways,” *Immunity*, 32(3), pp. 392–402. Available at: https://doi.org/10.1016/j.immuni.2010.03.001.
210. Nieminen, J. *et al.* (2008) “Role of Galectin-3 in Leukocyte Recruitment in a Murine Model of Lung Infection by *Streptococcus pneumoniae*,” *Journal of Immunology*, 180(4), pp. 2466–2473. Available at: https://doi.org/10.4049/jimmunol.180.4.2466.

211. Neuendorf, R. *et al.* (2016) “Depression and anxiety in patients with Inflammatory Bowel Disease: A systematic review,” *Journal of Psychosomatic Research*, 87, pp. 70–80. Available at: https://doi.org/10.1016/j.jpsychores.2016.06.001.
212. NHS England. (2022) “Getting it Right The First Time; IBD Pathway. Gastroenterology Version 1,” Available at: https://gettingitrightfirsttime.co.uk/wp-content/uploads/2022/01/Gastroenterology_2022-01-18_Pathway_IBD.pdf (last accessed 30/06/2023).
213. NHS England. (2023) “Consultant-led referral to treatment waiting times data 2022-23,” Available at: https://www.england.nhs.uk/statistics/statistical-work-areas/rtt-waiting-times/rtt-data-2022-23/ (last accessed 06/03/2023)
214. Noble, W.S. (2009) “How does multiple testing correction work?,” Nature Biotechnology, p. 1136. Available at: https://www.nature.com/articles/nbt1209-1135.pdf.
215. Noor, N.M. *et al.* (2024) “A biomarker-stratified comparison of top-down versus accelerated step-up treatment strategies for patients with newly diagnosed Crohn’s disease (PROFILE): a multicentre, open-label randomised controlled trial,” *The Lancet. Gastroenterology & Hepatology* [Preprint]. Available at: https://doi.org/10.1016/s2468-1253(24)00034-7.
216. Öhman, L. *et al.* (2021) “Fecal microbiota dynamics during disease activity and remission in newly diagnosed and established ulcerative colitis,” *Scientific Reports*, 11(1). Available at: https://doi.org/10.1038/s41598-021-87973-7.
217. Oksanen, F.J. et al. (2017) “Vegan: Community Ecology Package. R package Version 2.4-3,” Available at: https://CRAN.R-project.org/package=vegan
218. Oomizu, S. *et al.* (2012) “Galectin-9 suppresses Th17 cell development in an IL-2-dependent but Tim-3-independent manner,” *Clinical Immunology*, 143(1), pp. 51–58. Available at: https://doi.org/10.1016/j.clim.2012.01.004.
219. Page, M.J. *et al.* (2021) “The PRISMA 2020 statement: an updated guideline for reporting systematic reviews,” *BMJ*, p. n71. Available at: https://doi.org/10.1136/bmj.n71.
220. Paljetak, H.Č. *et al.* (2022) “Gut microbiota in mucosa and feces of newly diagnosed, treatment-naïve adult inflammatory bowel disease and irritable bowel syndrome patients,” *Gut Microbes*, 14(1). Available at: https://doi.org/10.1080/19490976.2022.2083419.
221. Palmela, C. *et al.* (2018) “Adherent-invasive *Escherichia coli* in inflammatory bowel disease,” *Gut*, 67(3), pp. 574–587. Available at: https://doi.org/10.1136/gutjnl-2017-314903.
222. Papa-Gobbi, R. *et al.* (2016) “A galectin-specific signature in the gut delineates Crohn’s disease and ulcerative colitis from other human inflammatory intestinal disorders.,” *PubMed*, 42(1), pp. 93–105. Available at: https://doi.org/10.1002/biof.1252.
223. Papa-Gobbi, R. *et al.* (2021) “Spatiotemporal regulation of galectin-1-induced T-cell death in lamina propria from Crohn’s disease and ulcerative colitis patients,” *Apoptosis*, 26(5–6), pp. 323–337. Available at: https://doi.org/10.1007/s10495-021-01675-z.
224. Paramsothy, S. *et al.* (2019) “Specific bacteria and metabolites associated with response to fecal microbiota transplantation in patients with ulcerative colitis,” *Gastroenterology*, 156(5), pp. 1440-1454.e2. Available at: https://doi.org/10.1053/j.gastro.2018.12.001.

225. Park, A.-M. *et al.* (2016) “Galectin-3 Plays an Important Role in Innate Immunity to Gastric Infection by Helicobacter pylori,” *Infection and Immunity*, 84(4), pp. 1184–1193. Available at: https://doi.org/10.1128/iai.01299-15.
226. Patil, I. (2018). “*ggstatsplot: 'ggplot2' Based Plots with Statistical Details*,” [R package]. Available at: https://CRAN.R-project.org/package=ggstatsplot.
227. Pereira, M.M. *et al.* (2018) “Glycans as Key Checkpoints of T Cell Activity and Function,” *Frontiers in Immunology*, 9. Available at: https://doi.org/10.3389/fimmu.2018.02754.
228. Pérez-Brocal, V. *et al.* (2015) “Metagenomic analysis of Crohn’s disease patients identifies changes in the virome and microbiome related to disease status and therapy, and detects potential interactions and biomarkers,” *Inflammatory Bowel Diseases*, 21(11), pp. 2515–2532. Available at: https://doi.org/10.1097/mib.0000000000000549.
229. Perler, B. *et al.* (2019) “Presenting symptoms in inflammatory bowel disease: descriptive analysis of a community-based inception cohort,” *BMC Gastroenterology*, 19(1). Available at: https://doi.org/10.1186/s12876-019-0963-7.
230. Pesole, P.L. et al. (2023) “90K/MAC-2 BP is a new predictive biomarker of response to infliximab therapy in IBD patients,” International Journal of Molecular Sciences, 24(4), p. 3955. Available at: https://doi.org/10.3390/ijms24043955.
231. Peyrin-Biroulet, L. *et al.* (2012) “Development of the first disability index for inflammatory bowel disease based on the international classification of functioning, disability and health,” *Gut*, 61(2), pp. 241–247. Available at: https://doi.org/10.1136/gutjnl-2011-300049.
232. Pigneur, B. *et al.* (2019) “Mucosal Healing and Bacterial Composition in Response to Enteral Nutrition Vs Steroid-based Induction Therapy-A Randomised Prospective Clinical Trial in Children With Crohn's Disease,” *Journal of Crohns and Colitis,* 13(7):846-855. Available at: https://doi:10.1093/ecco-jcc/jjy207.
233. Pope, C. (2023) “NHS waiting times: a government pledge,” *BMJ*, p. p71. Available at: https://doi.org/10.1136/bmj.p71.
234. Prescott, S.L. (2017) “History of medicine: Origin of the term microbiome and why it matters,” *Human Microbiome Journal*, 4, pp. 24–25. Available at: https://doi.org/10.1016/j.humic.2017.05.004.
235. Priya, S. *et al.* (2022) “Identification of shared and disease-specific host gene–microbiome associations across human diseases using multi-omic integration,” *Nature Microbiology*, 7(6), pp. 780–795. Available at: https://doi.org/10.1038/s41564-022-01121-z.
236. Procházková, N. *et al.* (2022) “Advancing human gut microbiota research by considering gut transit time,” *Gut*, 72(1), pp. 180–191. Available at: https://doi.org/10.1136/gutjnl-2022-328166.
237. Pu, D., Zhang, Z. and Feng, B.-S. (2022) “Alterations and potential applications of gut microbiota in biological therapy for inflammatory bowel diseases,” *Frontiers in Pharmacology*, 13. Available at: https://doi.org/10.3389/fphar.2022.906419.
238. Püngel, D. *et al.* (2020) “Bifidobacterium breve UCC2003 Exopolysaccharide Modulates the Early Life Microbiota by Acting as a Potential Dietary Substrate,” *Nutrients*, 12(4), p. 948. Available at: https://doi.org/10.3390/nu12040948.

239. Quince, C. *et al.* (2017) “Shotgun metagenomics, from sampling to analysis,” *Nature Biotechnology*, 35(9), pp. 833–844. Available at: https://doi.org/10.1038/nbt.3935.
240. Quraishi, M.N. (2020) “*Exploring interactions between host and gut microbiota in ulcerative colitis and primary sclerosing cholangitis associated inflammatory bowel disease: An appraisal through faecal microbiota transplantation and systems biology,”* University of Birmingham. Ph.D.
241. Quraishi, M.N. *et al.* (2023) “Establishing key performance indicators for inflammatory bowel disease in the UK,” *Frontline Gastroenterology*, p. flgastro-102409. Available at: https://doi.org/10.1136/flgastro-2023-102409.
242. R Core Team. (2021) “*R: A Language and environment for statistical computing*. (Version 4.1)” [Computer software]. Available at: https://cran.r-project.org.
243. Radjabzadeh, D. *et al.* (2020) “Diversity, compositional and functional differences between gut microbiota of children and adults,” *Scientific Reports*, 10(1). Available at: https://doi.org/10.1038/s41598-020-57734-z.
244. Radjabzadeh, D. *et al.* (2022) “Gut microbiome-wide association study of depressive symptoms,” *Nature Communications*, 13(1). Available at: https://doi.org/10.1038/s41467-022-34502-3.
245. Rausch, P. *et al.* (2023) “Microbial dynamics in newly diagnosed and treatment naïve IBD patients in the Mediterranean,” *Inflammatory Bowel Diseases*, 29(7), pp. 1118–1132. Available at: https://doi.org/10.1093/ibd/izad004.
246. Read, E., Curtis, M.A. and Neves, J.F. (2021) “The role of oral bacteria in inflammatory bowel disease,” *Nature Reviews Gastroenterology & Hepatology*, 18(10), pp. 731–742. Available at: https://doi.org/10.1038/s41575-021-00488-4.
247. Rees, N.P. *et al.* (2022) “Systematic review of donor and recipient predictive biomarkers of response to faecal microbiota transplantation in patients with ulcerative colitis,” *EBioMedicine*, 81, p. 104088. Available at: https://doi.org/10.1016/j.ebiom.2022.104088.
248. Regueiro, M. *et al.* (2018) “Reduced unplanned care and disease activity and increased quality of life after patient enrollment in an inflammatory bowel disease medical home,” *Clinical Gastroenterology and Hepatology*, 16(11), pp. 1777–1785. Available at: https://doi.org/10.1016/j.cgh.2018.04.007.
249. Ren, D. *et al.* (1996) “Mechanism of cholesterol reduction to coprostanol by Eubacterium coprostanoligenes ATCC 51222,” *Steroids*, 61(1), pp. 33–40. Available at: https://doi.org/10.1016/0039-128x(95)00173-n.
250. Riley, R.D. *et al.* (2020) “Calculating the sample size required for developing a clinical prediction model,” *The BMJ*, p. m441. Available at: https://doi.org/10.1136/bmj.m441.
251. Rimmer, P. et al. (2022) “P59 The pre-treatment gut microbiome at IBD diagnosis: early insights into the Birmingham inception cohort study,” Gut, p. A67.2-A68. Available at: https://doi.org/10.1136/gutjnl-2022-bsg.117.
252. Rinninella, E. *et al.* (2019) “What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases,” *Microorganisms*, 7(1), p. 14. Available at: https://doi.org/10.3390/microorganisms7010014.

253. Ripley, B., Venables, W. (2016) "*nnet: Feed-Forward Neural Networks and Multinomial Log-Linear Models*," [R package]. Retrieved from https://cran.r-project.org/package=nnet. (last accessed 04/01/2024).
254. Rodríguez, L.F., Ruigómez, A. and Panés, J. (2006) "Acute gastroenteritis is followed by an increased risk of inflammatory bowel disease," *Gastroenterology*, 130(6), pp. 1588–1594. Available at: https://doi.org/10.1053/j.gastro.2006.02.004.
255. Rohatgi A. (2021). "WebPlotDigitizer (Version 4.7)," [Computer software]. Retrieved from http://arohatgi.info/WebPlotDigitizer.
256. Rojas-Feria, M. *et al.* (2018) "Modulation of faecal metagenome in Crohn's disease: Role of microRNAs as biomarkers," World Journal of Gastroenterology, 24(46):5223-5233. Available at: https://doi:10.3748/wjg.v24.i46.5223.
257. Røseth, A. *et al.* (1992) "Assessment of the neutrophil dominating protein calprotectin in feces: a Methodologic study," *Scandinavian Journal of Gastroenterology*, 27(9), pp. 793–798. Available at: https://doi.org/10.3109/00365529209011186.
258. Roswell, M., Dushoff, J. and Winfree, R. (2021) "A conceptual guide to measuring species diversity," *Oikos*, 130(3), pp. 321–338. https://doi.org/10.1111/oik.07202.
259. Rowan, F.E. *et al.* (2009) "Sulphate-reducing bacteria and hydrogen sulphide in the aetiology of ulcerative colitis," *British Journal of Surgery*, 96(2), pp. 151–158. Available at: https://doi.org/10.1002/bjs.6454.
260. Sankaran, K. and Holmes, S. (2019) "Latent variable modeling for the microbiome," *Biostatistics*, 20(4), pp. 599–614. Available at: https://doi.org/10.1093/biostatistics/kxy018.
261. Sankaran, K. and Holmes, S. (2019) "Multitable Methods for Microbiome Data Integration," *Frontiers in Genetics*, 10. Available at: https://doi.org/10.3389/fgene.2019.00627.
262. Santucci, L. *et al.* (2003) "Galectin-1 suppresses experimental colitis in mice," *Gastroenterology*, 124(5), pp. 1381–1394. Available at: https://doi.org/10.1016/s0016-5085(03)00267-1.
263. Sauk, J.S. *et al.* (2023) "High Perceived Stress is Associated With Increased Risk of Ulcerative Colitis Clinical Flares," *Clinical Gastroenterology and Hepatology*, 21(3), pp. 741-749.e3. Available at: https://doi.org/10.1016/j.cgh.2022.07.025.
264. Sawczenko, A. (2003) "Presenting features of inflammatory bowel disease in Great Britain and Ireland," *Archives of Disease in Childhood*, 88(11), pp. 995–1000. Available at: https://doi.org/10.1136/adc.88.11.995.
265. Schirmer, M. *et al.* (2018) "Compositional and temporal changes in the gut microbiome of pediatric ulcerative colitis patients are linked to disease course," *Cell Host & Microbe*, 24(4), pp. 600-610.e4. Available at: https://doi.org/10.1016/j.chom.2018.09.009.
266. Schlichtner, S. *et al.* (2021) "Functional role of galectin-9 in directing human innate immune reactions to Gram-negative bacteria and T cell apoptosis," *International Immunopharmacology*, 100, p. 108155. Available at: https://doi.org/10.1016/j.intimp.2021.108155.
267. Schmidt, T.S. *et al.* (2019) "Extensive transmission of microbes along the gastrointestinal tract," *eLife*, 8. Available at: https://doi.org/10.7554/elife.42693.

268. Schroeder, K.W., Tremaine, W.J. and Ilstrup, D.M. (1987) “Coated oral 5-Aminosalicylic acid therapy for mildly to moderately active ulcerative colitis,” *The New England Journal of Medicine*, 317(26), pp. 1625–1629. Available at: https://doi.org/10.1056/nejm198712243172603.
269. Schumacher, G. *et al.* (1993) “A prospective study of first attacks of inflammatory bowel disease and non-relapsing colitis. Microbiologic findings,” *The Scandinavian Journal of Gastroenterology*, 28(12):1077-85. Available at: https://doi:10.3109/00365529309098313.
270. Schumacher, G. *et al.* (1995) “Virulence Properties of Escherichia coli Isolated from the Rectal Mucosa of Patients with First Attacks of Colitis,” *Microbial Ecology in Health and Disease*, *8*(1), 1–7. Available at: https://doi.org/10.3109/08910609509141375.
271. Selin, K.A., Hedin, C.R.H. and Villablanca, E.J. (2021) “Immunological networks defining the heterogeneity of inflammatory bowel diseases,” *Journal of Crohn S and Colitis*, 15(11), pp. 1959–1973. Available at: https://doi.org/10.1093/ecco-jcc/jjab085.
272. Seol, H. (2023). “*snowCluster: Multivariate Analysis*. (Version 6.8.3),” [jamovi module]. Available at: https://github.com/hyunsooseol/snowCluster.
273. Shah, R. *et al.* (2016) “Composition and function of the pediatric colonic mucosal microbiome in untreated patients with ulcerative colitis,” *Gut Microbes*, 7(5), pp. 384–396. Available at: https://doi.org/10.1080/19490976.2016.1190073.
274. Shapiro, J.S. *et al.* (2021) “Immunoglobulin A Targets a Unique Subset of the Microbiota in Inflammatory Bowel Disease,” *Cell Host Microbe*, 29(1), pp. 83-93.e3. Available at: https://doi.org/10.1016/j.chom.2020.12.003.
275. Shannon, C.E. and Weaver, W. (1963) *“The mathematical theory of communication,” University of Illinois Press,* 5:1-131.
276. Shaw, K.A. *et al.* (2016) “Dysbiosis, inflammation, and response to treatment: a longitudinal study of pediatric subjects with newly diagnosed inflammatory bowel disease,” *Genome Medicine*, 8(1). Available at: https://doi.org/10.1186/s13073-016-0331-y.
277. Shi, F. *et al.* (2012) “Dysregulated Tim-3 expression and its correlation with imbalanced CD4 helper T cell function in ulcerative colitis,” *Clinical Immunology*, 145(3), pp. 230–240. Available at: https://doi.org/10.1016/j.clim.2012.09.001.
278. Shi, Z. *et al.* (2018) “Decrease of galectin-3 in keratinocytes: A potential diagnostic marker and a critical contributor to the pathogenesis of psoriasis,” *Journal of Autoimmunity*, 89, pp. 30–40. Available at: https://doi.org/10.1016/j.jaut.2017.11.002.
279. Shkoda, A. *et al.* (2007) “Differential protein expression profile in the intestinal epithelium from patients with inflammatory bowel disease.,” *Journal of Proteome Research*, 6(3), pp. 1114–25. Available at: https://doi.org/10.1021/pr060433m.
280. Si, Y. *et al.* (2021) “Human galectin-16 has a pseudo ligand binding site and plays a role in regulating c-Rel-mediated lymphocyte activity,” *Biochimica Et Biophysica Acta - General Subjects*, 1865(1), p. 129755. Available at: https://doi.org/10.1016/j.bbagen.2020.129755.
281. Sila, S. *et al.* (2020) “Altered gut microbiota is present in newly diagnosed pediatric patients with inflammatory bowel disease,” *Journal of Pediatric*

Gastroenterology and Nutrition, 70(4), pp. 497–502. Available at: https://doi.org/10.1097/mpg.0000000000002611.
282. Silverberg, M.S. *et al.* (2005) "Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: report of a working party of the 2005 Montreal World Congress of Gastroenterology," *The Canadian Journal of Gastroenterology*, 19(suppl a), pp. 5A-36A. Available at: https://doi.org/10.1155/2005/269076.
283. Simpson E.H. (1949) "*Measurement of diversity," Nature,* 163: 688
284. Singh, S.R.K. *et al.* (2011) "Common symptoms and stressors among individuals with inflammatory bowel diseases," *Clinical Gastroenterology and Hepatology*, 9(9), pp. 769–775. Available at: https://doi.org/10.1016/j.cgh.2011.05.016.
285. Singh, V. *et al.* (2023) "Butyrate producers, 'The Sentinel of Gut': Their intestinal significance with and beyond butyrate, and prospective use as microbial therapeutics," *Frontiers in Microbiology*, 13. Available at: https://doi.org/10.3389/fmicb.2022.1103836.
286. Singmann, H. (2018). "*afex: Analysis of Factorial Experiments*," [R package]. Available at: https://cran.r-project.org/package=afex.
287. Sjöberg, F. *et al.* (2017) "Low-complexity microbiota in the duodenum of children with newly diagnosed ulcerative colitis," *PLoS ONE*, 12(10), p. e0186178. Available at: https://doi.org/10.1371/journal.pone.0186178.
288. Soucy, G. *et al.* (2012) "Clinical and pathological analysis of colonic Crohn's disease, including a subgroup with ulcerative colitis-like features," *Modern Pathology*, 25(2), pp. 295–307. Available at: https://doi.org/10.1038/modpathol.2011.120.
289. Somineni, H.K. *et al.* (2021) "Site- and Taxa-Specific Disease-Associated oral microbial structures distinguish inflammatory bowel diseases," *Inflammatory Bowel Diseases*, 27(12), pp. 1889–1900. Available at: https://doi.org/10.1093/ibd/izab082.
290. Splinter, M.J. *et al.* (2021) "Prevalence and determinants of healthcare avoidance during the COVID-19 pandemic: A population-based cross-sectional study," *PLOS Medicine*, 18(11), p. e1003854. Available at: https://doi.org/10.1371/journal.pmed.1003854.
291. Stapley, S. *et al.* (2017) "Clinical features of bowel disease in patients aged <50 years in primary care: a large case-control study," *British Journal of General Practice*, 67(658), pp. e336–e344. Available at: https://doi.org/10.3399/bjgp17x690425.
292. Stowell, S.R. *et al.* (2014) "Microbial glycan microarrays define key features of host-microbial interactions," *Nature Chemical Biology*, 10(6), pp. 470–476. Available at: https://doi.org/10.1038/nchembio.1525.
293. Suez, J. *et al.* (2022) "Personalized microbiome-driven effects of non-nutritive sweeteners on human glucose tolerance," *Cell*, 185(18), pp. 3307-3328.e19. Available at: https://doi.org/10.1016/j.cell.2022.07.016.
294. Tadbiri, S. *et al.* (2021) "The IBD-disk Is a Reliable Tool to Assess the Daily-life Burden of Patients with Inflammatory Bowel Disease," *Journal of Crohn's and Colitis*, 15(5), pp. 766–773. Available at: https://doi.org/10.1093/ecco-jcc/jjaa244.
295. Tanaka, M. and Riddell, R.H. (1990) "The pathological diagnosis and differential diagnosis of Crohn's disease.," *PubMed*, 37(1), pp. 18–31. Available at: https://pubmed.ncbi.nlm.nih.gov/2179090.

296. Tang, W. *et al.* (2020) “Effect of exclusive enteral nutrition on the disease process, nutrition status, and gastrointestinal microbiota for Chinese children with Crohn’s Disease,” *Journal of Parenteral and Enteral Nutrition*, 45(4), pp. 826–838. https://doi.org/10.1002/jpen.1938.
297. The jamovi project (2022). “*jamovi,”* (Version 2.3) [Computer Software]. Available at: https://www.jamovi.org.
298. Thiele, C. (2019) “*cutpointr: Determine and Evaluate Optimal Cutpoints in Binary Classification Tasks*,” [R package]. Retrieved from https://cran.r-project.org/package=cutpointr. (last accessed 04/01/2024).
299. Timms, V.J. *et al.* (2016) “The Association of Mycobacterium avium subsp. paratuberculosis with Inflammatory Bowel Disease,” *PLOS ONE*, 11(2), p. e0148731. Available at: https://doi.org/10.1371/journal.pone.0148731.
300. Tokarz, R. *et al.* (2019) “Characterization of stool virome in children newly diagnosed with moderate to severe ulcerative colitis,” *Inflammatory Bowel Diseases*, 25(10), pp. 1656–1662. Available at: https://doi.org/10.1093/ibd/izz099.
301. Travis, S. *et al.* (2013) “Reliability and initial validation of the ulcerative colitis endoscopic index of severity,” *Gastroenterology*, 145(5), pp. 987–995. Available at: https://doi.org/10.1053/j.gastro.2013.07.024.
302. Turner, D. *et al.* (2021) “STRIDE-II: An Update on the Selecting Therapeutic Targets in Inflammatory Bowel Disease (STRIDE) Initiative of the International Organization for the Study of IBD (IOIBD): Determining Therapeutic Goals for Treat-to-Target strategies in IBD,” *Gastroenterology*, 160(5), pp. 1570–1583. Available at: https://doi.org/10.1053/j.gastro.2020.12.031.
303. Turvill, J. *et al.* (2018) “Evaluation of the clinical and cost-effectiveness of the York Faecal Calprotectin Care Pathway,” *Frontline Gastroenterology*, 9(4), pp. 285–294. Available at: https://doi.org/10.1136/flgastro-2018-100962.
304. Ueno, A. *et al.* (2013) “Increased Prevalence of Circulating Novel IL-17 Secreting Foxp3 Expressing CD4+ T Cells and Defective Suppressive Function of Circulating Foxp3+ Regulatory Cells Support Plasticity Between Th17 and Regulatory T Cells in Inflammatory Bowel Disease Patients,” *Inflammatory Bowel Diseases*, 19(12), pp. 2522–2534. Available at: https://doi.org/10.1097/mib.0b013e3182a85709.
305. Umar, N. *et al.* (2022) “The association between inflammatory bowel disease and mental ill health: a retrospective cohort study using data from UK primary care,” *Alimentary Pharmacology & Therapeutics*, 56(5), pp. 814–822. Available at: https://doi.org/10.1111/apt.17110.
306. United States Food and Drug Administration. (2006) “Guidance for industry: patient-reported outcome measures: use in medical product development to support labeling claims: draft guidance,” *Health and Quality of Life Outcomes*, 4(1). Available at: https://doi.org/10.1186/1477-7525-4-79.
307. Van Andel, E.M. *et al.* (2020) “Systematic Review of Development and Content Validity of Patient-reported Outcome Measures in Inflammatory Bowel Disease: Do We Measure What We Measure?,” *Journal of Crohn’s and Colitis*, 14(9), pp. 1299–1315. Available at: https://doi.org/10.1093/ecco-jcc/jjaa057.
308. Van De Guchte, M., Mondot, S. and Doré, J. (2021) “Dynamic properties of the intestinal ecosystem call for combination therapies, targeting inflammation and microbiota, in ulcerative colitis,” *Gastroenterology*, 161(6), pp. 1969-1981.e12. Available at: https://doi.org/10.1053/j.gastro.2021.08.057.

309. Van Hul, M. et al. (2024) “What defines a healthy gut microbiome?,” *Gut*, 73(11), pp. 1893–1908. Available at: https://doi.org/10.1136/gutjnl-2024-333378.
310. Varma, S., Green, P.H. and Krishnareddy, S. (2020) “The distribution of gastrointestinal pathogens on stool PCR prior to the development of IBD,” *Journal of Clinical Gastroenterology*, 56(1), pp. e52–e57. Available at: https://doi.org/10.1097/mcg.0000000000001470.
311. Vatanen, T. *et al.* (2016) “Variation in microbiome LPS immunogenicity contributes to autoimmunity in humans,” *Cell*, 165(4), pp. 842–853. Available at https://doi.org/10.1016/j.cell.2016.04.007.
312. Vatn, S. *et al.* (2020) “Faecal microbiota signatures of IBD and their relation to diagnosis, disease phenotype, inflammation, treatment escalation and anti-TNF response in a European Multicentre Study (IBD-Character),” *Scandinavian Journal of Gastroenterology*, 55(10), pp. 1146–1156. Available at: https://doi.org/10.1080/00365521.2020.1803396.
313. Vavricka, S.R. *et al.* (2012) “Systematic evaluation of risk factors for diagnostic delay in inflammatory bowel disease,” *Inflammatory Bowel Diseases*, 18(3), pp. 496–505. Available at: https://doi.org/10.1002/ibd.21719.
314. Veltkamp, C. *et al.* (2011) “Apoptosis of regulatory T lymphocytes is increased in chronic inflammatory bowel disease and reversed by anti-TNF treatment,” *Gut*, 60(10), pp. 1345–1353. Available at: https://doi.org/10.1136/gut.2010.217117.
315. VERBI Software. (2021) “MAXQDA 2022,” [Computer programme], VERBI Software, Berlin, Germany. Available at: https://www.maxqda.com
316. Volarevic, V. *et al.* (2019) “Galectin-3 Regulates Indoleamine-2,3-dioxygenase-Dependent Cross-Talk between Colon-Infiltrating Dendritic Cells and T Regulatory Cells and May Represent a Valuable Biomarker for Monitoring the Progression of Ulcerative Colitis,” *Cells*, 8(7), p. 709. Available at: https://doi.org/10.3390/cells8070709.
317. Walsh, A. *et al.* (2018) “Defining faecal calprotectin thresholds as a surrogate for endoscopic and histological disease activity in ulcerative colitis—a prospective analysis,” *Journal of Crohn’s and Colitis*, 13(4), pp. 424–430. Available at: https://doi.org/10.1093/ecco-jcc/jjy184.
318. Wang, A. *et al.* (2024) “The oral-gut microbiome axis in inflammatory bowel disease: from inside to insight,” *Frontiers in Immunology*, 15. Available at: https://doi.org/10.3389/fimmu.2024.1430001.
319. Wang, L. *et al.* (2020) “Gut Microbial Dysbiosis in the irritable bowel Syndrome: A Systematic Review and Meta-Analysis of Case-Control Studies,” *Journal of the Academy of Nutrition and Dietetics*, 120(4), pp. 565–586. Available at: https://doi.org/10.1016/j.jand.2019.05.015.
320. Wang, S. *et al.* (2019) “Fecal bacteria from ulcerative colitis patients downregulate Tim-3-mediated inhibitory effects on monocytes in vitro,” *Microbial Pathogenesis*, 128, pp. 147–152. Available at: https://doi.org/10.1016/j.micpath.2018.12.043.
321. Wang, W. *et al.* (2021) “Galectin-9 targets NLRP3 for autophagic degradation to limit inflammation,” *Journal of Immunology*, 206(11), pp. 2692–2699. Available at: https://doi.org/10.4049/jimmunol.2001404.
322. Wang, X. *et al.* (2021) “Characteristics of fecal microbiota and machine learning strategy for fecal invasive biomarkers in pediatric inflammatory bowel

disease," *Frontiers in Cellular and Infection Microbiology*, 11. Available at: https://doi.org/10.3389/fcimb.2021.711884.
323. Wang, Y. et al. (2021) "Microbial and metabolic features associated with outcome of infliximab therapy in pediatric Crohn's disease," *Gut Microbes*, 13(1):1-18. Available at: https://doi:10.1080/19490976.2020.1865708.
324. Waugh, N. *et al.* (2013) "Faecal calprotectin testing for differentiating amongst inflammatory and non-inflammatory bowel diseases: systematic review and economic evaluation," *Health Technology Assessment*, 17(55). Available at: https://doi.org/10.3310/hta17550.
325. Weinstein, L. (1961e) "Bacteriologic Aspects of Ulcerative Colitis," *Gastroenterology*, 40(2), pp. 323–330. Available at: https://doi.org/10.1016/s0016-5085(61)80124-8.
326. Weinstock, G.M. (2012) "Genomic approaches to studying the human microbiota," *Nature*, 489(7415), pp. 250–256. Available at: https://doi.org/10.1038/nature11553.
327. White, K. and Makin, A. (2022) "P149 Prioritising colonoscopy by faecal immunochemical test result does not delay the diagnosis of inflammatory bowel disease," *Journal of Crohn S and Colitis*, 16(Supplement_1), p. i228. Available at: https://doi.org/10.1093/ecco-jcc/jjab232.277.
328. Whittaker, R.H. (1960) "Vegetation of the Siskiyou Mountains, Oregon and California," *Ecological Monographs*, 30(3), pp. 279–338. Available at: https://doi.org/10.2307/1943563.
329. Whittaker, R.H. (1972) "EVOLUTION AND MEASUREMENT OF SPECIES DIVERSITY," *Taxon*, 21(2–3), pp. 213–251. Available at: https://doi.org/10.2307/1218190.
330. Wickham, H. et al. (2018). "*ggplot2: Create Elegant Data Visualisations Using the Grammar of Graphics*," [R package]. Available at: https://CRAN.R-project.org/package=ggplot2.
331. Willing, B.P., Russell, S.L. and Finlay, B.B. (2011) "Shifting the balance: antibiotic effects on host–microbiota mutualism," *Nature Reviews Microbiology*, 9(4), pp. 233–243. Available at: https://doi.org/10.1038/nrmicro2536.
332. Wopereis, H. *et al.* (2014) "The first thousand days - intestinal microbiology of early life: establishing a symbiosis," *Pediatric Allergy and Immunology*, 25(5), pp. 428–438. Available at: https://doi.org/10.1111/pai.12232.
333. Wu, Y. *et al.* (2021) "Accuracy of the Hospital Anxiety and Depression Scale Depression subscale (HADS-D) to screen for major depression: systematic review and individual participant data meta-analysis," *BMJ*, p. n972. Available at: https://doi.org/10.1136/bmj.n972. Erratum in: BMJ. 2021 May 19;373:n1231.
334. Xu, J. *et al* (2018), "5-aminosalicylic acid alters the gut bacterial microbiota in patients with ulcerative colitis," *Frontiers in Microbiology*, 9:1274:1–13. Available at: https://doi:10.3389/fmicb.2018.01274.
335. Xu, J. *et al.* (2019) "Alteration of fungal microbiota after 5-ASA treatment in UC patients," *Inflammatory Bowel Diseases*, 26(3), pp. 380–390. https://doi.org/10.1093/ibd/izz207
336. Yan, Y. *et al.* (2023) "Bacteroides uniformis-induced perturbations in colonic microbiota and bile acid levels inhibit TH17 differentiation and ameliorate colitis developments," *Npj Biofilms and Microbiomes*, 9(1). Available at: https://doi.org/10.1038/s41522-023-00420-5.

337. Yu, T. *et al.* (2020) “Serum galectins as potential biomarkers of inflammatory bowel diseases,” *PLOS ONE*, 15(1), p. e0227306. Available at: https://doi.org/10.1371/journal.pone.0227306.
338. Zhao, X. *et al.* (2011) “Evaluation of P38 MAPK pathway as a molecular signature in ulcerative colitis,” *Journal of Proteome Research*, 10(5), pp. 2216–2225. Available at: https://doi.org/10.1021/pr100969w.
339. Zhu, C. *et al.* (2005) “The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity,” *Nature Immunology*, 6(12), pp. 1245–1252. Available at: https://doi.org/10.1038/ni1271.
340. Zhu, S. *et al.* (2022) “Composition and diverse differences of intestinal microbiota in ulcerative colitis patients,” *Frontiers in Cellular and Infection Microbiology*, 12. Available at: https://doi.org/10.3389/fcimb.2022.953962.
341. Zitomersky, N.L. *et al.* (2013) “Characterization of Adherent Bacteroidales from Intestinal Biopsies of Children and Young Adults with Inflammatory Bowel Disease,” *PLoS ONE*, 8(6), p. e63686. Available at; https://doi.org/10.1371/journal.pone.0063686.
342. Zuberi, R.I. *et al.* (2004) “Critical role for galectin-3 in airway inflammation and bronchial hyperresponsiveness in a murine model of asthma,” *American Journal of Pathology*, 165(6), pp. 2045–2053. Available at: https://doi.org/10.1016/s0002-9440(10)63255-5.

Conference Abstracts and publications during PhD

Submitted manuscripts

- The gut microbiome at the onset of inflammatory bowel disease: A systematic review of the literature, meta-analysis of diversity data and unified bioinformatic synthesis of sequencing data. **Submitted to Lancet Gastroenterology and Hepatology. Undergoing first peer review. 1st author.**
- The IBD-disk accurately assesses disability and psychological burden at IBD diagnosis and predicts adverse outcomes in UC during the first year of treatment. **Submitted to Alimentary Pharmacology and Therapeutics. Undergoing first peer review. 1st author.**
- Oral vancomycin induces remission of colitis in primary sclerosing cholangitis and is associated with multiple changes in host-microbiome-metabolome interactions. **Submitted to Journal of Crohn's and Colitis. Revision due for submission. 3rd author.**

Peer reviewed publications

- **Rimmer P,** Cheesbrough J, Harris J, Love M, Tull S, Iqbal A, Regan-Komito D, Cooney R, Hazel K, Sharma N, Dietrich T, Chapple I, Quraishi MN & Iqbal TH. Optimising triage of urgent referrals for suspected IBD: results from the Birmingham IBD inception study. Frontline Gastroenterology. **2024** Mar 12. Epub 2024 Mar 12.

Rimmer et al. Optimising Triage.pdf

- Saviano A, Schettino A, Iaccarino N, Mansour AA, Begum J, Marigliano N, Raucci F, Romano F, Riccardi G, Mitidieri E, d'Emmanuele di Villa Bianca R, Bello I, Panza E, Smimmo M, Vellecco V, **Rimmer P**, Cheesbrough J, Zhi Z, Iqbal TH, Pieretti S, D'Amore VM, Marinelli L, La Pietra V, Sorrentino R, Costa L, Caso F, Scarpa R, Cirino G, Randazzo A, Bucci M, McGettrick HM, Iqbal AJ, Maione F. A reverse translational approach reveals the protective roles of Mangifera indica in inflammatory bowel disease. J Autoimmun. **2024** Apr;144:103181

Saviano et al. Reverse translational approach.pdf

- Saviano A, Manosour AA, Raucci F, Merlino F, Marigliano N, Schettino A, Wahid M, Begum J, Filer A, Manning JE, Casillo GM, Piccolo M, Ferraro MG, Marzano S, Russomanno P, Bellavita R, Irace C, Amato J, Alfaifi M, **Rimmer P**, Iqbal T, Pieretti S, Vellecco V, Caso F, Costa L, Giacomelli R, Scarpa R, Cirino G, Bucci M, McGettrick HM, Grieco P, Iqbal AJ, Maione F. New biologic (Ab-IPL-IL-17) for IL-17-mediated diseases: identification of the bioactive sequence (nIL-17) for IL-17A/F function. Ann Rheum Dis. **2023** Nov;82(11):1415-1428.

Saviano et al. Il-17 mediated diseases..pdf

- **Rimmer P**, Howell G, Mahgoub S*, et al*. Every colon shouldn't have a silver lining: an atypical case of colitis caused by a 'commensal' organism. *Frontline Gastroenterology* 2023;**14:**352-353.

Rimmer et al inside view.pdf

Invited reviews and book chapters

- **Rimmer P**, Iqbal TH. Prognostic modelling in IBD. Best Practice & Research Clinical Gastroenterology,2023;67:101877

Prognostic modelling paper.pdf

- **Rimmer P,** Cooney R. (2024). Impact of Pregnancy and Childbirth on Pre-existing Bowel Conditions. In: Sultan, A.H., Thakar, R., Lewicky-Gaupp, C. (eds) Pelvic Floor, Perineal, and Anal Sphincter Trauma During Childbirth. Springer, Cham. 2024

Impact of pregnancy on existing bowel conditons - final 29.6.pdf

Conference abstracts

- **P Rimmer**, J Cheesbrough, Hazel K, R Horniblow, D Regan-Komito, R Cooney, AJ Iqbal, I Chapple, N Sharma, T Iqbal. Baseline microbial profiles predict early failure of mesalazine in new onset ulcerative colitis. *Gut* 2024;**73:**A130-A131.

Mesalazine failure.pdf

- K Hazel, **P Rimmer,** M Love, E Smith, J Goh, N Sharma, T Iqbal, S Samani, R Cooney. Shifting paradigms: a single trust experience of combination biologic or small molecule therapy for IBD. *Gut* 2024;**73:**A121-A122.

Shifting paradigms.pdf

- **P Rimmer**, G Scott, MN Quraishi, M Gordon, K Hazel, R Cooney, F Zhang, G Hold, T Iqbal, R Hansen. Is gut microbiome diversity important at IBD onset? A systematic review of the literature and meta-analysis of alpha diversity data. *Gut* 2024;**73:**A114-A115.

Diversity BSG.pdf

- J Cheesbrough, **P Rimmer,** N Sharma, N Quraishi, A Ahmed, T Iqbal. The positioning and acceptability of faecal microbiota transplantation as an IBD treatment, across a multi-ethnic IBD patient cohort. *Gut* 2024;**73:**A106-A107.

FMT acceptability.pdf

- **P Rimmer,** J Cheesbrough, R Horniblow, S Tull, A Beggs, R Cooney, N Sharma, D Regan-Komito, AJ Iqbal, T Iqbal, MN Quraishi. Sa1883 INCREASED ABUNDANCE AND ACTIVITY OF SUTTERELLA WADSWORTHENSIS AT DISEASE ONSET, ALONGSIDE DEPLETION OF SHORT CHAIN FATTY ACID PRODUCING CLOSTRIDIALES, ASSOCIATES WITH INCREASED USE OF ADVANCED THERAPIES IN THE FIRST TWO YEARS OF IBD DIAGNOSIS. Gastroenterology 2024;166(5):S-564

Sutterella ddw.pdf

- **P Rimmer**, G Scott, MN Quraishi, M Gordon, K Hazel, R Cooney, F Zhang, G Hold, T Iqbal, R Hansen. Sa1887 IS DIVERSITY IMPORTANT IN THE GUT MICROBIOME AT IBD ONSET? A SYSTEMATIC REVIEW OF THE LITERATURE AND META-ANALYSIS OF ALPHA-DIVERSITY DATA. Gastroenterology 2024;166(5):S-565-S-566.

Diversity DDW.pdf

- **P Rimmer**, J Cheesbrough, R Horniblow, S Tull, N Sharma, R Cooney, A Beggs, D Regan-Komito, A Iqbal, T Iqbal, M N Quraishi, P060 Increased metabolic activity of faecal Sutterella Wadsworthensis at IBD onset is associated with an increased risk of escalation to biologic therapies - Results from the Birmingham Inception Cohort Study, *Journal of Crohn's and Colitis*, Volume 18, Issue Supplement_1, January **2024**, Pages i329–i330

Sutterella ecco.pdf

- **P Rimmer**, J Cheesbrough, M Ibrahim, K Hazel, A Javed, A Iqbal, D Regan-Komito, S Tull, M Love, R Cooney, M N Quraishi, T Iqbal, P391 A not so innocent bystander: The prevalence and burden of anaemia at IBD onset, *Journal of Crohn's and Colitis*, Volume 18, Issue Supplement_1, January **2024**, Pages i820–i82
- Quraishi N, Ferguson J, McInnes R, Cheesbrough J, Sharma N, Cooney R, **Rimmer P**, van Schaik W, Iqbal T, Trivedi P. Oral vancomycin induces remission in PSC-IBD, which is associated with a reduction in bile salt hydrolase and colonic amine oxidase activity. Journal of Hepatology **2023**;78:S123-124.

oral vancomycin.pdf

- **Rimmer P**, Cheesbrough J, Horniblow R*, et al.* P84 Microbial associations with disease activity at adult IBD onset. *Gut* 2023;**72:**A96-A97.

microbial associations.pdf

- **Rimmer P**, Ibrahim M, Cooney R*, et al.* P70 Body and soul: the psychosocial disease burden of patients attending an adult IBD inception clinic. *Gut* 2023;**72:**A84-A85.
- **Rimmer P**, Cheesbrough J, Quraishi MN*, et al.* P69 Shortening the journey to diagnosis in Crohn's disease? Characteristics and outcomes of a prospectively collected inception cohort. *Gut* 2023;**72:**A84.

Body soul AND Journey to diagnosis.pdf

- **Rimmer P**, Cheesbrough J, Horniblow R*, et al.* P83 Can Faecal Microbiome composition changes be associated with increased disability at IBD onset, irrespective of mucosal inflammation?. *Gut* 2023;**72:**A94-A96.

Microbiome disability.pdf

- **Rimmer P**, Ibrahim M, Cooney R*, et al.* P71 Confronting the elephant in the room: can the IBD disk adequately assess psychological burden and disease activity in suspected IBD? *Gut* 2023;**72:**A85-A86.

Elephant in the room.pdf

- **Rimmer P**, Cheesbrough J, Quraishi MN et al. P126 Ask twice: The importance of repeated faecal calprotectin testing prior diagnostic colonoscopy in an adult inception cohort, *Journal of Crohn's and Colitis*, Volume 17, Issue Supplement_1, February 2023, Pages i291–i293

Ask twice.pdf

- **Rimmer P**, Cheesbrough J, Iqbal A et al. P923 The pre-treatment gut microbiome at adult IBD inception: Initial signals from the Birmingham inception cohort, *Journal of Crohn's and Colitis*, Volume 17, Issue Supplement_1, February 2023, Pages i1032–i1033

Inception microbiome ecco.pdf

- Stephenson B, **Rimmer P**, Smith A*, et al*. P68 Higher generic tacrolimus dosing may be required to achieve the same level and 12-month outcomes as innovator tacrolimus in liver transplantation. *Gut* 2022;**71:**A82-A83.

tacrolimus.pdf

- **Rimmer P**, Cheesbrough J, Quraishi MN*, et al*. P320 Developing an IBD inception pathway: a streamlined pathway to IBD diagnosis in the COVID era. *Gut* 2022;**71:**A195-A196.

Inception pathway.pdf

- **Rimmer P**, Horniblow R, Cheesbrough J*, et al*. P59 The pre-treatment gut microbiome at IBD diagnosis: early insights into the Birmingham inception cohort study. *Gut* 2022;**71:**A67-A68.

Early insights.pdf

- **P Rimmer**, Cheesbrough J, Palmer L et al. P155 Incidence and significance of vitamin D deficiency at Inflammatory Bowel Disease inception in the COVID era, *Journal of Crohn's and Colitis*, Volume 16, Issue Supplement_1, January 2022, Pages i231–i232

Vit D.pdf

- S Shariff, Sharma N, Quraishi MN, Iqbal T, Cooney R, **Rimmer P**. P193 Correlation of the IBD Disk scores with diagnosis of IBD, Faecal calprotectin and Endoscopic scores in patients presenting to a rapid access IBD inception clinic, *Journal of Crohn's and Colitis*, Volume 16, Issue Supplement_1, January 2022, Page i255.

Disk ecco.pdf

Appendix A

Systematic review and meta-analysis – Search strategy, ROBINS-E assessments, thematic analysis included studies table

Electronic repository

Shared folder hosting original files below

Password: ThesiS.Oct

https://1drv.ms/f/c/2161c93d62c1cb40/EkSItXSboIBKk-MuO5pHp0wBKKYpyTiLJU9ngvaIyVNhnA?e=9phJOW

Appendix B

MaAsLin2 – Differential abundance for 16S data below significance thresholds.

Electronic repository

Shared folder hosting original bioinformatic outputs below

Password: ThesiS.Oct

https://1drv.ms/f/c/2161c93d62c1cb40/EhLkn1XYjddAmkSXnV0t4zABV5gHo1ItZu5fNtaHzyMoqA?e=GGXtwN

Appendix C

TOPIC analysis of the microbiome at IBD onset.

Electronic repository

Shared folder hosting original bioinformatic outputs below

Password: ThesiS.Oct

https://1drv.ms/f/c/2161c93d62c1cb40/EvfC3PhKW4FBkM_uF7FwV7QBKt0oeMzjivW2C7NuItJwBg?e=P0nuYn

Appendix D

MaAsLin2 – Differential abundance for Shotgun Metagenomics data below significance thresholds

Electronic repository

Shared folder hosting original bioinformatic outputs below

Password: ThesiS.Oct

https://1drv.ms/f/c/2161c93d62c1cb40/EjBsNauZA35Am7J8O4ji6BgBGaKWSiBLbNMVut8EfMs9EA?e=RJf3eB

Appendix E

MaAsLin2 – Differential Meta-CYC pathway activity derived from Shotgun Metagenomic data below significance thresholds

Electronic repository

Shared folder hosting original bioinformatic outputs below

Password: ThesiS.Oct

https://1drv.ms/f/c/2161c93d62c1cb40/EiOA8_AVRQZKuLsKXD3hDq8Bo7a0sglbUciOEtCRIhyP_g?e=OK6JPv

Appendix F

Analyses from pooled 16S sequencing of publicly available IBD datasets

Electronic repository

Shared folder hosting original bioinformatic outputs below

Password: ThesiS.Oct

https://1drv.ms/f/c/2161c93d62c1cb40/EhevOpiw355FvOHyalOHEAEBJTAfv2QCHpuyT7IxFgzGEQ?e=sToyd0

Appendix G

Indices from Phyloseq analysis of Galectin 16S data

Electronic repository

Shared folder hosting original bioinformatic outputs below

Password: ThesiS.Oct

https://1drv.ms/f/c/2161c93d62c1cb40/EhF2MSPeME1GtWMrZIwufzYBWAuSz3rjZQwlahgP3O3AFQ?e=zgUPN9