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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

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Abstract

Inflammatory bowel disease (IBD) has progressively become a global epidemic and now affects nearly 0.5% of the Western population. The aetiological factors that initiate and drive mechanisms associated with IBD remain unclear. A cure has been even more elusive. Changes in the gut microbial diversity and profiles in individuals with this disease is a characteristic feature, however a causal relationship has yet to be proven. In my PhD I have attempted to explore host-microbiota interactions and its influence on mechanisms of ulcerative colitis (UC) and primary sclerosing cholangitis associated inflammatory bowel disease (PSC-IBD).

Patients with UC have a greater abundance of *Clostridiaceae* at inflamed compared to non-inflamed sites. Immunophenotyping demonstrated significantly higher proportions of colonic mucosal Th17 and IL-17 producing CD4 cells in patients with UC and PSC-IBD compared to healthy controls. Through an open label study (STOP-Colitis pilot phase), I demonstrated that faecal microbiota transplantation (FMT) resulted in a clinical response in 47% of patients (8/17; intention to treat). This response was associated with a significant increase in colonic mucosal regulatory T cells (Treg), effector memory Tregs, gut homing Tregs and IL-10 producing CD4 T cells population along with a concurrent decrease in Th17, IL-17 producing CD4 T cells and CD8 populations. Colonic mucosal transcriptomics revealed that responders to FMT had significant downregulation of antimicrobial defence and proinflammatory immunological pathways and an increase in butanoate metabolic pathways compared to both baseline and non-responders. Finally, through a multi-omic exploration of colonic mucosal biology, I demonstrated that the gene expression profiles in patients with PSC-IBD was significantly different to UC and was associated with dysregulation of bile acid homeostasis and signalling in association with colonic dysbiosis.

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CHAPTER 1

Introduction

1.1 Host immune - microbiota interactions in IBD – associative or causal?

1.1.1 Introduction

Inflammatory bowel disease (IBD) is a chronic, immune-mediated gastrointestinal condition that affects over 1 million of the UK population (1). It broadly comprises two diseases; ulcerative colitis (UC) and Crohn's disease (CD). Patients typically present with constellation of symptoms that include chronic diarrhoea, rectal bleeding and abdominal pain as a consequence of chronic inflammation (2). The incidence and prevalence of IBD markedly increased over the last 50 years and in the last decade it has become one of the most prevalent gastrointestinal diseases. Although the definitive cause of IBD remains yet unknown, substantial progress has been made in the recent years in exploring the pathogenesis of this disease. Traditionally IBD was considered to be an autoimmune disease. It is now increasingly accepted that it is the result of a dysregulated immune response against specific environmental triggers in genetically predisposed individuals (3).

Studies of IBD genetics have identified over 200 loci that explain a modest 8%–13% of disease susceptibility risk variance highlighting the importance of non-genetic / environmental drivers of disease. Although IBD emerged in the Western countries in the latter half of the 20th century, the progressive appearance of IBD in developing nations in the last 25 years suggests that these epidemiological shifts are related to lifestyle changes and industrialisation (4). The distinct genetic background of these emerging populations in contrast to IBD in Caucasian populations within the Western world, the lack of replication of some of the risk loci identified in these populations, and the rapid rise in incidence paralleling urbanisation and changing lifestyles underscore a crucial role of the environment in pathogenesis of IBD (5-7). Changes in dietary

habits, use of antibiotics, hygiene status including exposure to microbes and pollution been implicated as potential environmental risk factors for IBD. This critical significance of environmental influences is further supported by growing recognition of the fundamental role of the gut microbiota in the development and progression of inflammation in IBD (8). This is further highlighted in studies exploring IBD risk within immigrants. In the early 1990s Probert and colleagues identified that the incidence of ulcerative colitis in first-generation and second-generation South Asian migrants to the UK was similar to the native UK population, and significantly greater than the incidence in their countries of origin (9).

Environmental exposures in industrialized societies appear to contribute to IBD aetiology (10). Although several of these environmental risk factors have been explored, none of them have consistently or fundamentally have helped explain mechanisms that trigger and drive IBD. The hygiene hypothesis was first proposed by Strachan and colleagues to explain the rising incidence of autoimmune conditions in urbanised communities (11). This postulates that with greater industrialization and urbanization of society children have a significantly reduced exposure to microbes, such that infections later in life may trigger an abnormal host immune response due to reduced immunological tolerance. Consequently, early-life disruption in the intestinal microbiota could influence the development of IBD later in life. Several epidemiological studies in the Western world have supported the hygiene hypothesis and the role of the intestinal microbiota, including a modulated risk of IBD associated with antibiotic use in childhood, breast-feeding and urban versus rural residence (12-14). This was outlined in an early seminal nested case–control study from the University of Manitoba IBD cohort where 58% of paediatric patients with IBD received an antibiotic in their first year of life compared with only 39% of the controls (15).

Cigarette smoking is one of the most reliably studied environmental determinant of IBD. Specific to Crohn's disease, smoking increases its risk of onset, need for early surgery and postoperative recurrence, whilst smoking cessation improves both short and long term clinical outcomes (16). On the contrary, epidemiological studies suggests non-smokers or ex-smokers are at an increased risk of developing ulcerative colitis, although the evidence is contested. Smoking might contribute to the development of IBD by potentially influencing the gut microbiota as those with Crohn's disease who smoke show a dysbiosis (17).

Another important epidemiological risk factor appears to be differences in dietary composition associated with urbanisation. Most epidemiological studies of diet and IBD have concentrated on macronutrients, and by relying on a retrospective case control design have been susceptible to numerous limitations and bias (18). Nevertheless, the most consistent macronutrient association has been an inverse association with dietary fibre and IBD (19). A prospective cohort study observed that women who consumed significantly higher proportional of fibre intake over long term had a 40% reduction in risk of Crohn's disease(20). Several protective mechanisms have been suggested including metabolism of soluble fibre (from fruits and vegetables) by the gut bacteria to short-chain fatty acids that promote transcription of anti-inflammatory immune responses (21).

1.1.2 Gut microbiota

The gut microbiota exists as a complex multi-cellular community that lives synergistically with its host and is increasingly considered to be an important partner in health (22). This community consisting of bacteria, viruses, phages, fungi, yeast, archaea and other protists plays a crucial

role in influencing host physiology in health and disease. The gastrointestinal tract provides a complex, integrated and open ecosystem that contains at least 10^{14} microorganisms belonging to over 2,000 species and 12 different phyla. This associated microbiome contains vastly more genes than the human DNA. The healthy gut microbiota is primarily dominated by the phyla Firmicutes and Bacteroidetes, followed by Proteobacteria and Actinobacteria to a lesser extent. Collectively they act in symbiosis to contain and supress expansion of pathologic organisms, facilitate metabolism of dietary substrates and environmental chemicals and importantly contribute towards intestinal epithelial cell renewal and development of the immune system. In fact, numerous metabolic activities and signals that the microbiota deploys are complementary to those of the host, reinforcing that humans are composite organisms.

The gut microbiota exhibits an immense diversity and is shaped throughout life by numerous and incompletely elucidated factors that include host genetics, age, geographical and socio-economic factors, diet and disease. Although the microbiome is largely temporally stable and resistant to perturbations in an adult, it does not exist in a single static state, but rather as a dynamic equilibrium. Consequently, in health the gut microbiota may transiently shift following an environmental trigger such as antibiotics or a gastrointestinal infection before usually recovering to its baseline. This plasticity exhibited by the microbiota suggests that not only can its composition and function be modified throughout life by extrinsic and intrinsic factors but also makes it a viable therapeutic target for treatment of gut microbiota mediated diseases.

1.1.3 Gut microbiota in IBD

Shifts in the composition and function of gut microbiota (also known as dysbiosis) have been associated with a multitude of immune mediated chronic diseases of the gastrointestinal tract including IBD and primary sclerosing cholangitis (23). Initial studies implicating the role of bacteria in the pathogenesis of IBD have attempted to identify a single possible culprit as the initiator of the inflammatory cascade seen in IBD. Although bacteria including *Mycobacterium avium* subsp *paratuberculosis*, *Helicobacter*, *Campylobacter* and adherent / invasive *Escherichia coli* have been proposed as direct contributors in IBD aetiopathogenesis no single microorganism has yet reliably been implicated. With the advent of next generation sequencing, the focus in the last decade shifted with the recognition that the gut microbiota is altered as a whole in IBD. The possibility of this dysbiosis being a trigger in the development and progression of IBD has been one the major areas of focus in recent research in IBD.

Cross sectional cohort studies profiling gut microbiota consistently report shifts in specific microbial taxa. These notably include a decrease in genera of the phylum Bacteroidetes (e.g. *Barnesiella* and *Alistipes*) and Firmicutes (e.g. *Faecalibacterium, Oscillibacter, Ruminococcus*) and an increase in Proteobacteria comprising species such as *Escherichia coli* and other members of *Enterobacteriaceae* family are found to be increased in patients with IBD when compared to healthy individuals. Additionally, a specific reduction in microorganisms such as *Bifidobacterium adolescentis* and *Faecalibacterium prausnitzii* that are associated with clinical remission has been demonstrated. (8, 24-27). These alterations appear to be represented by reduced abundance of butyrate-producing species that include *Blautia faecis*, *Roseburia inulinivorans, Ruminococcus torques* and *Faecalibacterium prausnitzii* and an increase in

sulfate-reducing bacteria, such as *Desulfovibrio* and other species with pro-inflammatory properties such as adhesion-invasive E. coli (AIEC) in IBD (28-30).

Although bacteria dominate microbial communities, viruses, archaea, and fungi also play pivotal roles in maintaining the gut homeostasis. However, the viral and fungal compositions within the gut microbiome remain relatively uncharacterised so far, due to their lower abundances, curated reference databases as well as lack of robust bioinformatics tools and pipelines (31). The gut virome includes diverse commensal and pathogenic viruses that have abilities to infect host cells as well as other microbes, both avenues can directly affect the host's health. Although there is a high inter-individual variation, the gut virome is primarily dominated by bacteriophages. A lesser component of the community of gut viromes includes retroviruses, prophages and eukaryotic viruses (32). The gut virome, specifically bacteriophages, can elicit chronic inflammation by infecting and killing the host cell as well as beneficial / immunomodulatory populations of gut bacteria. The decreasing richness (alpha diversity) of gut bacteria occurring in patients with IBD is well established. In contrast, however, the faecal virome composition is increased in richness in IBD compared to healthy controls (33, 34). Specifically, and consistently, the population of Caudovirales bacteriophages were increased and more diverse relative to healthy controls.

In addition to the virome, the gut mycobiome (or fungeome) is relatively unexplored. This is the collection of the fungal community and their respective genomes associated with the gut with metagenomics sequencing studies suggesting that these account of approximately 0.1% of the gut microbiome (35). Fungal genera usually detected include Candida, Saccharomyces, and Galactomyces, however the potential roles played by these microbes in the human gut is poorly

understood (36). The gut mycobiome of patients with IBD has been characterised by reduced fungal diversity and an imbalance in community populations relative to healthy control (37). It is clear that interdependence between bacterial community, virome, and mycobiome exist and any alteration in the gut microbial composition may have the potential to impact host health. For example, bacteriophages can lyse commensal or pathogenic bacteria and drive bacterial evolution in the gut, moulding the gut microbiome (38). In turn, the gut bacterial community may inhibit pathogen colonization through competition, produce secondary metabolites, and facilitate development of immune responses.

Collectively and consistently, these findings strongly indicate that the alteration of gut microbiota is associated with the pathogenesis of IBD. Whether this is a primary or secondary event and if the mucosal immune response is appropriate or exaggerated are two of the fundamental questions that remains to be answered (39). From data accruing in studies exploring multiple aspects of host immune interactions with gut microbiota and their manipulation, key mechanisms of causality are progressively being uncovered with a view to discovering novel treatment targets for IBD (40).

1.1.4 Host immune microbiota relationships govern IBD

The gut faces the exceptional challenge of maintaining intestinal immune tolerance and host mutualism to the vast and diverse commensal microbiota while mounting appropriate defence to pathogens. Host immune cells in conjunction with the intestinal barrier manage this through a variety of immunological mechanisms that in addition to the cellular and humoral immune responses include mucus secretion, immunoglobulin A (IgA), and antimicrobial peptides. This

immune homeostasis in turn facilitates the maintenance of a relatively stable gut microbial community while limiting the colonisation of pathogenic organisms. Dysregulation of many facets of the mucosal immune homeostasis is the cardinal feature that drives disease in IBD. Genome wide association studies in IBD demonstrate variants across candidate genes involved in multiple immune pathways including antigen sensing, immune cell trafficking and pathogen handling (41, 42). Despite the close relationship between host and microbiome in IBD, confident identification of associations between host genetic variants and microbiome composition remains challenging. Links between genetic variants and gut microbes. Variant in NOD2, for example are associated with abundance of *Faecalibacterium prausnitzii*, family *Enterobacteriaceae* and genus *Roseburia* (43). NOD2 is a intracellular host sensor of bacterial infection that recognises the prokaryotic cell wall component muramyl dipeptide (MDP) and triggers a proinflammatory cytokine response (44). Risk variants in IBD generally implicate a core set of functionally related host microbiome pathways that represent vulnerabilities to gut inflammation (45). These primarily include four categories: (1) innate cytokine pathways and antimicrobial defence sensors and (2) antibacterial effector mechanisms, (3) adaptive pathways including antigen presentation and cytokine production and (4) epithelial barrier function (46).

With the alarming rate of increase in the global incidence and prevalence of IBD, it appears more likely that environmentally derived immune triggers are driving the development of IBD rather than a seeming unlikely rapid increase in the pool of these gene variants. Regions in Asia, which are currently viewing the highest incidence of IBD, are genetically distinct from the traditionally high-risk Western countries (4). Epidemiological studies have identified a number of environmental influences associated with the development of IBD ranging from events at birth, exposure to antibiotics in early life and a Western diet as shown in Figure 1 - 1 (10, 20, 47). Although no direct causality can be associated with these factors in the disruption of mucosal

immune homeostasis, it is clear that these individually have a major effect on the development and maintenance of gut microbiota. Studies have consistently shown that patients with IBD have significant dysbiosis compared to those without disease. Expansion of potential pathogens as well as global reduction in the symbiotic species and compositional diversity have been consistently described. For example members of the phylum Firmicutes, specifically *Faecalibacterium prausnitzii,* have been shown to be reduced in both stool and mucosal biopsies while the phylum Proteobacteria comprising species such as *Escherichia coli* and other members of *Enterobacteriaceae* family are found to be increased in patients with IBD when compared to healthy individuals (8, 24-27). Whether this is a primary or secondary event and if the mucosal immune response is appropriate or exaggerated are two of the fundamental questions that remains to be answered (39). While a single causative agent has remained elusive, the origin of IBD likely to be a consequence of an aberrant host immunological response (influenced partly by genetic predisposition) to an environmentally dictated shift in the gut microbiota.

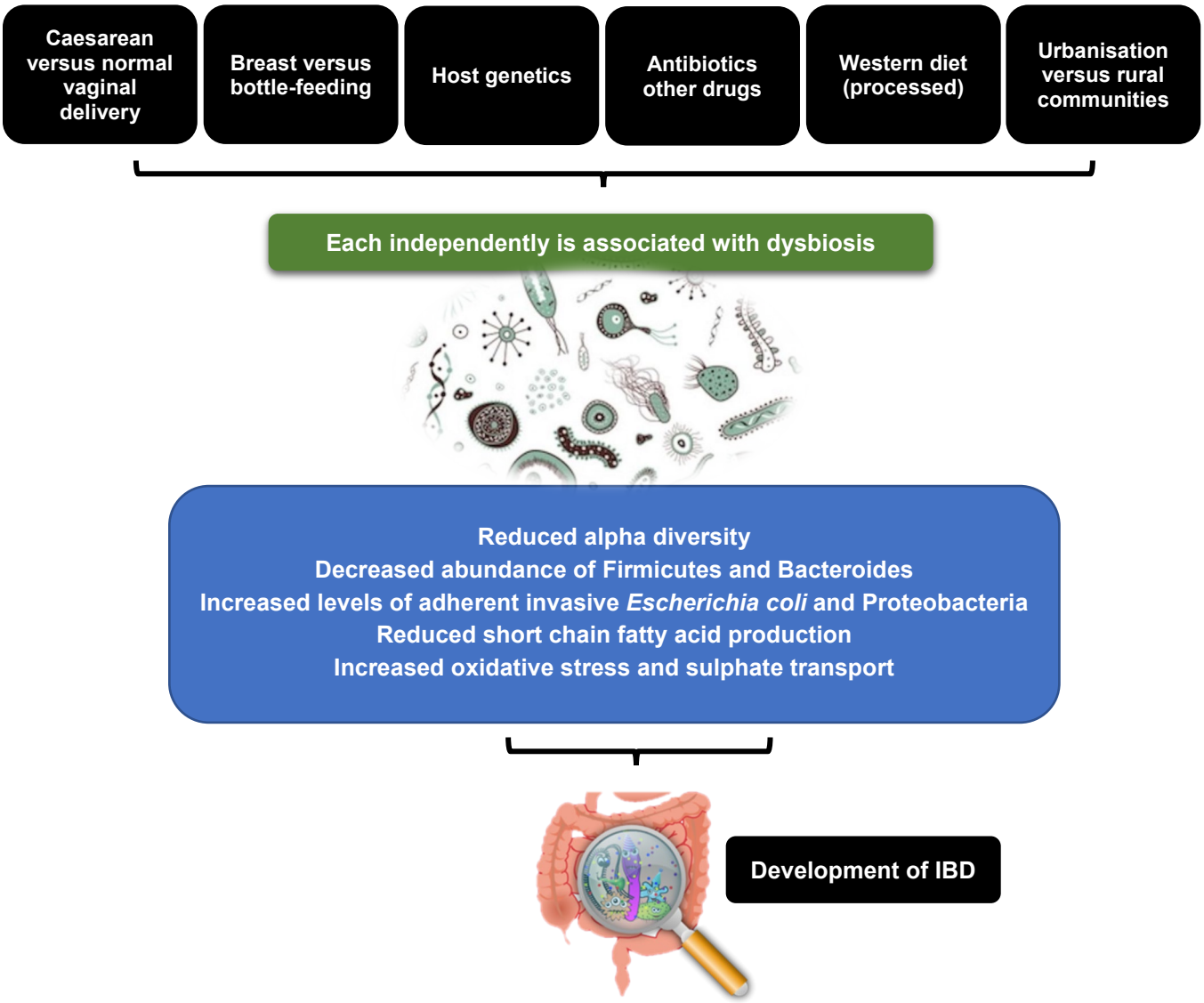


Figure 1 - 1 : Summary of risk factors and gut microbial changes associated with the development of IBD

Influences on the gut microbiome throughout life starting from conception to adulthood contribute to dysbiosis and development of IBD. The gut microbiome is susceptible to the influence of host genetics and environmental influences such as mode of childbirth, early antibiotic use and Western diet. Epidemiological studies have consistently linked urbanisation with increasing IBD prevalence. This is particularly apparent in countries that have witnessed industrialisation and westernisation in the last few decades. The resultant shifts in microbial taxonomic composition and function appear to contribute to the development of intestinal inflammation in IBD.

Figure adapted from Ananthakrishnan A et al. Nat Gastro Hep 2015

1.1.5 Evidence of the causal role of dysbiosis for pro-inflammatory innate and adaptive immune responses in IBD

There is considerable evidence demonstrating complex dynamic and bidirectional mechanistic relationships between gut microbiota and immune mediated inflammatory responses which contribute to the pathogenesis of IBD.

Innate immunity

The gut mucosal innate immune response is ostensibly directed towards one or many foreign antigens and pathogens (48, 49). Microbial sensing occurs through toll-like receptors (TLRs) and NOD-like receptors (NLRs) that recognise pathogen-associated molecular patterns (PAMPs) such as flagellin, lipopolysaccharide (LPS) and muramyl dipeptide (MDP). Gene polymorphisms of these receptors along with increased expression in innate immune cells have been described in patients with IBD (50-52). Specific TLRs are associated with induction of either inflammatory or anti-inflammatory responses. Mucosal macrophages and dendritic cells demonstrate upregulation of TLR2, TLR4 as well as CD40 and chemokine receptor CCR7 in patients with UC and CD compared to healthy controls (53). This consequently promotes inflammation through increased production of pro-inflammatory cytokines such as IL-1, IL-6, TNF-α, IL-18 and members of the IL-12 family. Colonisation of germ-free (GF) mice with a complex gut microbiota augments expression of TLR2 and is partly reversed by broad-spectrum antibiotics (54). This effect appears to be bidirectional as expression a flagellin sensing transmembrane receptor, TLR5, regulates the composition and localization of the intestinal microbiota (55). TLRs also have the ability to promote intra-epithelial cells (IECs) and Paneth cells to produce anti-microbial proteins such as RegIIIβ/γ that can kill Gram-positive bacteria

following microbial-epithelial contact (56, 57). Patients with IBD have increased expression of Reg proteins suggestive of compensatory defensive mechanisms against enteric pathogens (58).

Further recently discovered components of the innate immune response that play an important role in maintenance of gut mucosal homeostasis and tissue repair includes the Mucosal-Associated Invariant T (MAIT) cells, Natural Killer T Cells (NKT) cells and the family of Innate Lymphoid Cells (ILC). MAIT cells are unconventional T cells that express an invariant T cell receptor (TCR) α chain (59). Studies have observed a protective role for MAIT cells in TSA (trinitrobenzene sulfonic acid) colitis in mice (60). MAIT cells frequencies are higher in the inflamed colonic mucosa and lower in the peripheral blood of IBD patients compared to healthy controls (61). Moreover, MAIT cell in the peripheral blood of IBD patients are characterized by an increased proliferative state in vivo with over-expression of Ki67 and are at increased susceptibility to apoptosis due to over-expression of activated caspases (62). MAIT cells secrete high levels of IL-17A, TNF-α, and IL-22 in patients with IBD. The correlation between the frequency of gut tissue resident MAIT cells and low percentage of gut homing marker α4β7 integrin expressing in the peripheral blood MAIT cells suggests recruitment to the inflamed mucosa (62). Its role in regulating host microbiota immune responses are now increasingly relevant. MAIT cells can be activated by cells that are infected with different bacterial species such as Mycobacterium tuberculosis, Escherichia coli and Staphylococcus aureus (63). Furthermore, expansion of MAIT cells in the periphery and the acquisition of a memory phenotype is observed in germ-free mice reconstituted with different bacterial species suggesting the role of commensal flora in MAIT induction (64) (65).

Natural Killer T Cells NKT cells share phenotypic and functional features with both conventional NK cells including the presence of CD3 and TCR. NKT cells recognise antigens expressed on antigen-presenting cells. Lipid antigens derived from bacteria and fungi can be presented by CD1d molecules and recognised by the TCRs on NKT cells. NKT cells can also be activated by recognition of lipids as well as pro-inflammatory cytokines generated during infection. NKT cells are classifed into type I NKT cells (invariant NKT cells) or type 11 NKT cells based on their TCR. A protective role for NKT cells sustained by Th1 response has been described in DSS colitis, TSA colitis and T cells adoptive transfer murine (66). Conflicting data on CD1d have been described in IBD patients. One study showed high levels of CD1d (antigen-presenting molecule that restricts NKT cells) in the inflamed ileum and cecum of patients with IBD, whereas absence of CD1d has been reported in the IBD intestinal epithelium (67). Furthermore, NKT cells that secrete IL-13 from lamina propria have been found to be significantly reduced in UC in comparison with CD and controls (68). Increased frequencies of type II NKT cells producing IL-13 have been observed in UC lamina propria, their function in IBD remains ulcer (69). It is however possible that its role may be influenced by various local factors including cytokine mileu, the nature of the antigens presented the type of stimulatory antigen-presenting cells, the cytokine milieu, and the gut microbiota.

These innate immune cells belong to the lymphoid lineage and have certain functional similarities with the adaptive CD4 T helper cell populations but do not possess an antigen specific T or B cell receptor. On the basis of their phenotypic and functional heterogeneity, ILCs have been primarily classified in three subsets: ILC1, express the Th1 cell transcription factor T-bet and produce the Th1 cytokines TNF-α and IFN-γ; ILC2 defined by the Th2 cells transcription factor GATA3 and by the release of the Th2 specific cytokines IL-5 and IL-13; ILC3 positive for the Th17 transcription factor RORγt and that produce IL-22 without or with IL-17A. Patients with

IBD have altered abundances and functionality of the different ILC subsets in the gut compared to healthy individuals (70, 71). Many key genes related to ILC3 (innate counterpart to Th17 cells) biology were identified as IBD risk loci in GWAS studies and are involved in the IL-23/IL-17 pathway (72). ILC3 subpopulations have been shown to shape microbial communities by either modifying epithelial function or the functional properties of other cells that influence microbiota composition. Certain tryptophan based microbial metabolites were shown to directly control functionality of ILC3 through activation of ligand-dependent transcription factors in particular aryl hydrocarbon receptor (AhR) (73). Mice with a deletion of MHCII in ILC3 developed spontaneous colitis in the presence of commensal bacteria (74).

Adaptive immunity

In addition to the initial contact between the environment and the immune system through the innate immune pathway, the adaptive immune system also plays a crucial role in the progression of chronic inflammation in IBD (75, 76). CD4+ T cell subsets play central roles in the formation of cytokine networks in IBD pathogenesis. Seminal work in the early 90s demonstrated that adoptive transfer of CD4 T cells failed to induce colitis in RAG -/- mice (deficient in B and T cells) with reduced bacterial load or raised under germ free conditions (77, 78). Furthermore CD4 T cells isolated from mice that develop spontaneous colitis were strongly reactive to MHC class II antigens from gut commensals but not to epithelial or food antigens (79). Adoptive transfer of bacterial-antigen-activated CD4+ T cells from these mice into severe combined immunodeficient (SCID) mice was able to induce colitis when activated by gut microbial antigens. These findings suggested that intestinal inflammation is driven by resident gut bacteria.

The plasticity and adaptability of T helper (Th) cells based on host environmental factors makes them highly relevant in the development and pathogenesis of IBD (Figure 1 - 2). Historically, T cell subsets were described as either Th1 cells that secrete interferon gamma (IFN-γ), essential for eradication of intracellular pathogens or Th2 cells that secrete IL-4, 5 and 13 and play an essential role to the response against parasites and fibrosis process. Consequently, based on cytokine production, Crohn's disease was traditionally considered to be primarily a Th1 condition and UC characterised as a Th2 mediated disease (3).

The discovery of IL-17 producing Th17 cells and immunomodulating regulatory T cells (Treg) led to the refinement of this paradigm. Among these subsets of CD4+ T cells, Th17 cells have been shown to have critical roles in mucosal defence and in the pathogenesis of autoimmune diseases (80, 81). These cells, along with expression of IL-17, shown to be highly enriched in the intestinal lamina propria of patients with IBD (82). Th17 cells are not found in the intestines of germ-free reared mice suggesting that gut bacteria are possibly responsible for generation of this immune subset (83). Segmented filamentous bacteria (SFB) or *Candidatus Savagella* have been shown to induce RORγt+ Th17 subsets in mice and this is likely to occur through a mechanism independent of TLR, NOD, and ATP-signalling (83-85). SFB is also a potent stimulus of the mouse intestinal IgA response and induces the recruitment of intraepithelial lymphocytes (IEL) (86-88). Although SFBs are present in rodents, chicken and fish, direct evidence of human SFB is lacking. Apart from data in a handful of qPCR, 16s rRNA and histology studies, bioinformatics searches through the Human Microbiome Project (HMP) database and other human metagenomic databases for human SFB genes in been negative(89). More recently, other human symbiont bacterial species such as *Bifidobacterium adolescentis* have been shown to drive Th17 differentiation in mice (90, 91).

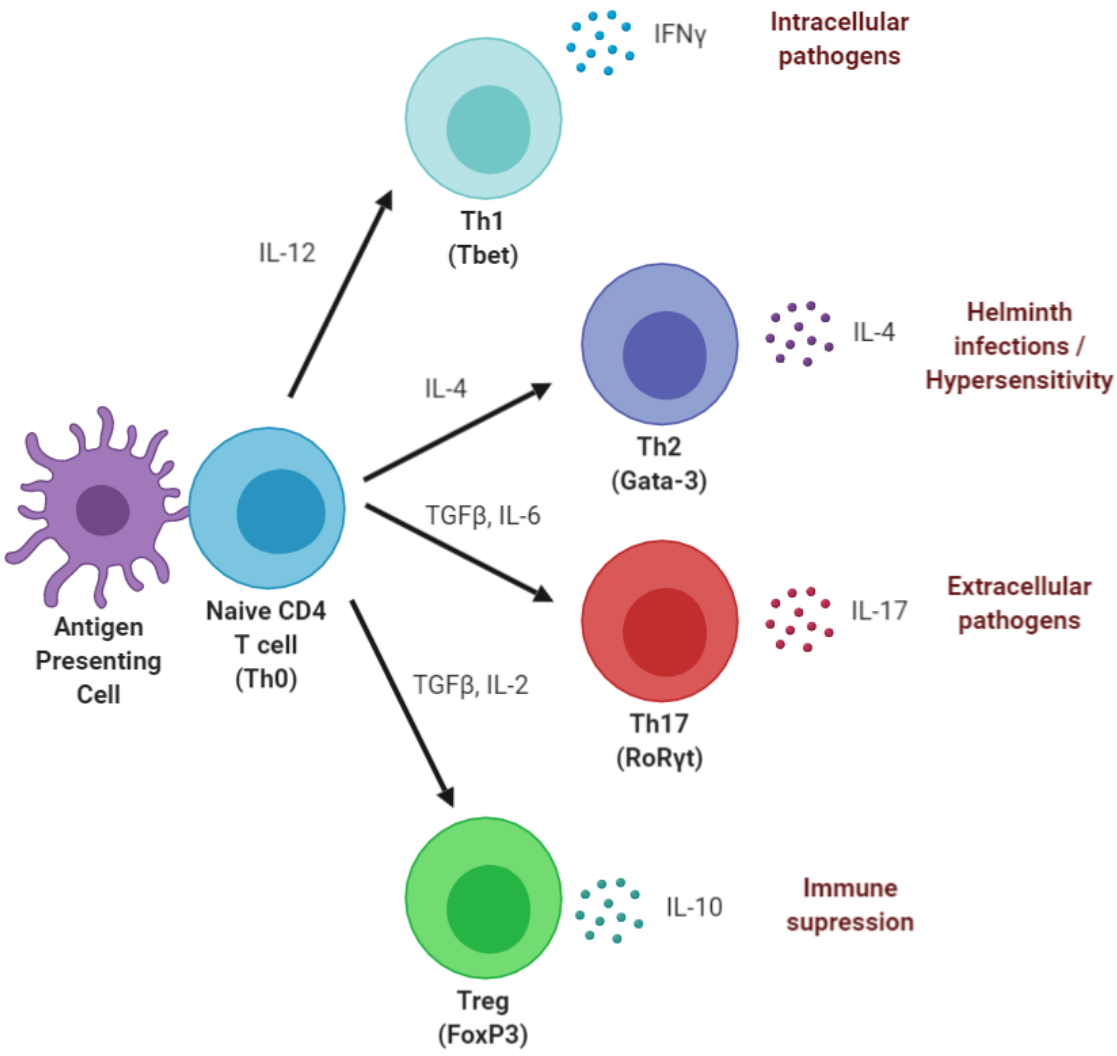


Figure 1 - 2 : Summary of key CD4 T cell subsets

The different CD4 + subsets are generated from the naive T cells by the different cytokines. For each T helper subset a distinct transcription factor has been identified as shown in brackets. Each T helper subset produces a different set of key cytokines and is important during the defence against certain pathogens or immune disorders. Moreover, under the influence of different cytokines, Th cells, in particular Th1, Th2 and Th17 central memory cells at early stages of differentiation are highly plastic and can switch lineage. In contrast, effector memory cells are more differentiated, resistant to plasticity and are more likely to become polyfunctional.

Breakdown in the homeostatic adaptive immune inflammatory and regulatory mechanisms appears to play a fundamental role in the development of IBD. The development of tolerance to gut commensals is fundamental to the induction and maintenance of a host-microbial mutualistic T cell response thereby limiting microbe-triggered gut inflammation (92). IL-10-/-, IL-2-/- and IL-2R-/- mice that have dysfunctional or reduced Treg frequencies develop colitis and are commonly used models of IBD (93). Although germ free IL-10-/- mice do not develop colitis, introduction of specific strains of bacteria in such as Lactobacillus plantarum 299V into specific pathogen-free IL-10-/- mice had a protective effect against the development of colitis whereas strains of Enterococcus faecalis induced colitis (94-96). Interestingly and perhaps paradoxically multiple reports have demonstrated that Tregs represent a greater fraction of the lamina propria mononuclear cells in the intestines of IBD patients compared to healthy controls (97, 98). Tregs are even more common in actively inflamed than non-inflamed mucosa. Moreover, IBD patients with high-proinflammatory cytokines have been shown to demonstrate an increased prevalence of dual lineage with IL-17 secreting Foxp3 expressing CD4+ T cell subsets in the circulation and tissue. Whether these Tregs have inherent functional deficits in immunoregulatory activity or are Tregs demonstrating Th17 plasticity is yet to be determined (99, 100). Mechanisms by which microbial stimuli results in Treg-Th17 conversion via TLR2 signalling have been highlighted (101).

Certainly, the importance of microbiota in influencing Treg mediated intestinal homeostasis has been described in several key studies. Transfer of specific Clostridia strains derived from human stool into germ free mice induced a threefold increase in Tregs (102). This Treg increase was significantly higher with the transfer of 30 strains compared to a single strain highlighting the role of microbial diversity in host immune responses in IBD (103). These strains were able to induce important anti-inflammatory molecules including IL-10, TGF- β1, CTLA-4 and inducible

T-cell co-stimulator (ICOS) in addition to the production of immunoregulatory short chain fatty acids (SCFA). Species from these SCFA producing *Clostridiales* species, have been shown to be reduced in the gut microbiota in patients with IBD, supporting the gut microbiota mediated Treg functionality hypothesis (104). A recent pivotal study demonstrated that transfer of microbiota from IBD patients into germ-free mice significantly increased numbers of intestinal Th17 cells and decreased numbers of RORγt+ Treg cells compared to microbiota from healthy individuals (105). Furthermore, colonisation with microbiota derived from IBD patients exacerbated colitis in a T cell transfer Rag1 −/− mouse model and the disease status correlated with increase in microbiota-induced proportions of Th17 and RORγt+ Treg cells compared to mice colonised with healthy donor microbiotas. These findings collectively highlight the role of microbiota in determining intestinal Th17 and RORγt+ Treg cell compartments as an important mechanism of pathogenesis in IBD.

A variety of innate and adaptive immune mechanisms are known to influence immunoglobulin responses to the intestinal microbiota (106, 107). Increased infiltration of intestinal mucosal plasma cells and mucosal immunoglobulin levels provide further compelling evidence for gut mucosal- microbial interaction in the pathogenesis of IBD. High levels of mucosal IgG directed against commensal bacterial antigens have been described in the gut in IBD and these appear to be principally directed against the bacterial cytoplasmic rather than the membrane proteins (108, 109). Patients with UC appear to have higher serum IgG responses to species including *Peptostreptococcus anaerobius* strains, *Enteroccocus faecalis*, *Streptococcus bovis* and specific bacteria from the Clostridia class and these antibodies greatly enhanced the respiratory burst in polymorphonuclear neutrophils in response to bacterial species (110). A key study that performed 16S rRNA sequencing of IgA coated intestinal microbiota (IgA-seq) isolated from stool in IBD patients discovered that 35 species were uniquely highly coated in patients with IBD

and often this was independent of differences in abundances (111). These IgA inducing members of the intestinal microbiota cultured from IBD patients exacerbated DSS colitis in germ free mice thereby highlighting its causal role in susceptibility to colitis.

1.2.1 Exploring gut microbiota manipulation for treatment of IBD

As no single causative trigger has been yet identified, similar to most immune-mediated diseases, treatment of IBD is primarily directed towards suppression of host immunological consequences. Current treatments for IBD are focused on counteracting multiple facets of these immune pathways. Drugs blocking pleiotropic pro-inflammatory pathways such as steroids, immunomodulatory drugs, anti-TNF agents, the Th1/Th17 axis via IL-12/23 blockage and leukocyte trafficking via anti-integrins and signalling molecules are the mainstay of medical management of IBD (112). These agents are fairly effective with steroid free clinical remission rates ranging from 39.7% for combination therapy with infliximab (anti-TNF) and azathioprine (thiopurine) to 18% for tofactinib (targets JAK/STAT signalling pathway) (113) (114). With increasing evidence of a dysregulated intestinal microbiome being a likely immunological trigger for inflammation in IBD, exploring therapeutic manipulation of gut microbiome is an attractive strategy to identify new treatment options and even unravel the underlying cause for IBD.

Microbial manipulation in IBD has been evaluated through several approaches. A fairly untargeted approach using antibiotics has only proved successful in treating inflammation that occurs in the distal small bowel that has been refashioned into a pouch following removal of the large bowel for colitis - known as pouchitis (115). There is limited evidence for the role of antibiotics in mild to moderate Crohn’s disease with questionable long term benefits (116).

Certainly, antibiotic related adverse events, unknown long-term consequences associated with alterations in the microbiome and the emergence of antimicrobial resistant genes makes this strategy of limited attractiveness. Targeted modulation of gut microbiota has been explored with the use of probiotics. These are live microorganisms that mediate their effects in treating IBD through potentially upregulating anti-inflammatory immune pathways. Results in clinical trials in IBD have been mixed, with meta-analyses only showing benefit in modest prevention of relapse only for UC when used in conjunction with 5-aminosalicylates and moderate quality evidence highlighting its role in prevention of pouchitis (117, 118).

One of the likely reasons for the lack of success with antibiotics and probiotics is that these approaches tend to assume that microbial triggers for IBD follow Koch's postulates and attempt an untargeted change of the dysbiosis. However, if we assume that the underlying mechanisms stimulating IBD is a shift in the gut microbial community towards an ecosystem that induces and maintains host pro-inflammatory pathways then re-establishing this community towards a 'healthy' population of microbes should in effect restore immunological homeostasis.

1.2.2 Faecal microbiota transplantation

Faecal microbiota transplantation (FMT) is the transfer of a processed stool obtained from a healthy donor into a patient with the aim to correct the underlying dysbiosis by attempting to restore the intestinal microbial community (119). An summary of production of FMT from stool is shown in Figure 1 - 3. Fermented faecal suspensions as therapy for gastrointestinal diseases was first described in the 4th century by Chinese physicians / alchemists Ge Hong and Li Shizen. In the current medical era, the first reported use of FMT was for the treatment of

pseudomembranous colitis in 1958. It now seems likely that these patients were colonised by an overgrowth of toxigenic *Clostridiodes difficile* as is commonly seen in patients (usually elderly and immunocompromised individuals) who have been treated with broad spectrum antibiotics. This results in *Clostridiodes difficile* infection (CDI) and about a third of patients will develop recurrent or persistent disease refractory to usual first line antibiotics while a third have recurrent episodes.

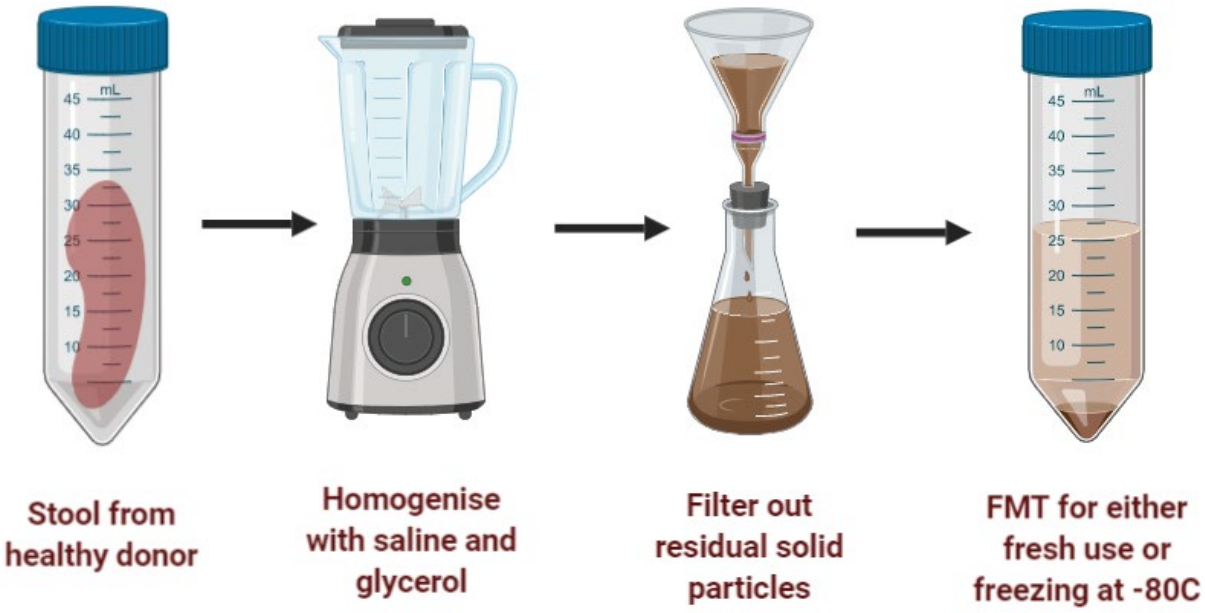


Figure 1 - 3 : Summary of production of FMT

Stool donations collected from healthy donors are processed within six hours of defaecation. Stool weighing at least 50g is mixed with preservative-free sterile 0.9% saline and 10% glycerol (for cryoprotection) in a ratio of 1:5. Stool is then homogenised and filtered. Traditionally this was done using a lab blender and a sieve. However as FMT is now labelled as a medicinal product by regulatory bodies, in order to comply with Good Manufacturing Procedure standards a sterile, single-use closed homogenisation and filtration system is recommended (such as sterile filter bags inside a laboratory paddle homogeniser). The filtrate is now classified as FMT and can either be used as a fresh infusion or frozen at -80C for later use (with a current shelf life of up to 6 months).

1.2.3 Protocol for production of FMT within a licenced setting

Preparation of medicinal FMT

Use of FMT for clinical trials requires compliance with Good Manufacturing Practice (GMP) required for Medicines and Health Regulatory Authority (MHRA) licencing (120). However, to develop and, the FMT facility processes and policies were embedded within a Quality Management System, overseen by a Qualified Person and developed to meet the requirements outlined within The Orange Guide. Procedures and control measures taken to ensure compliance with the Orange Guide and MHRA licencing at the UoBMTC are available online (121).

FMT donor selection and screening

Donors were healthy un-related anonymous volunteers, ≥18 and <50 years of age, with a Body Mass Index ≥18·5 and ≤25, who had not received antibiotics in the preceding three months. Screening was via health, social and travel questionnaire, clinical assessment (undertaken by an independent clinician) and microbiological testing (Figure 1 - 4). Health, social and travel exclusion criteria are presented in the supplementary information. FMT was quarantined until donor screening results were completed, health questionnaires had been reviewed and receipt of written confirmation of donor eligibility was received from the independent clinician.

Human Immunodeficiency Virus (Ag/Ab combo)		Hepatitis A Virus (IgM)
Hepatitis B Virus (HBsAG and anti HBc)		Hepatitis E Virus (IgM)
Hepatitis C Virus (anti-HCV-ab)		EBNA (IgG, IgM)
Syphilis total Ab screen (specific ELISA)		Norovirus (PCR)
CMV (IgM, IgG)		Rotavirus antigen (PCR)
*HTLV 1-2	*If indicated	*Strongyloides* (ELISA)
C. difficile (PCR)		*Helicobacter pylori* (Stool antigen)
Shigella sp. (PCR)		*Salmonella* species (PCR)
Escherichia coli O157 (c)		*Campylobacter* species (PCR)
Microsporidia (PCR)		*Cryptosporidium* (PCR, microscopy)
ESBL, VRE, CPE and MRSA (rectal culture)		*Giardia* (PCR)
Ova, Cyst and Parasite Microscopy: *Entamoeba histolytica, Entamoeba coli, Cyclospora, Isospora (*and others subject to travel risk assessment).		

Figure 1 - 4 : Blood and stool microbiology screening of donors.

Screening protocols updated since the publication of BSG guidelines in 2018. All microbiological tests are performed by a UKAS accredited microbiology laboratory.

FMT preparation

All FMT was prepared within UoBMTC. FMT processing was under aerobic conditions in a containment level two laboratory, within a sole use class two microbiological safety cabinet which was decontaminated before and after use using chlorine dioxide-based disinfectant (Tristel Solutions Ltd, UK) and ultra violet light (254 Nm, 30 minutes). Faeces were processed <6 hours post defecation. Macroscopic examination of donated stool was initially performed to ensure a Bristol Stool type 2–5, brown in colour and contained no macroscopic blood or mucus. FMT material was prepared by combining 90 ± 5 g of faeces from a single donor with either 150 ml IV grade 0·9% Saline (fresh FMT) or 150 ml IV grade 0·9% Saline containing 10% v/v glycerol (frozen FMT) in a sterile Nasco Whirl-Pak filter bag (Labs UK Ltd, UK) as shown in Figure 1 - 5. This was homogenised in a Mix-1 stomacher (AES Chemunex, France) for two

minutes (Figure 1). The filtrate was either transferred to an enteral feeding syringe and used immediately (fresh FMT) or stored in Biotite™ Containers (Alpha Laboratories, UK) in 60 ml aliquots at -80°C for up to 24 weeks (frozen FMT). FMT reference samples from every batch were retained and stored at -80°C.

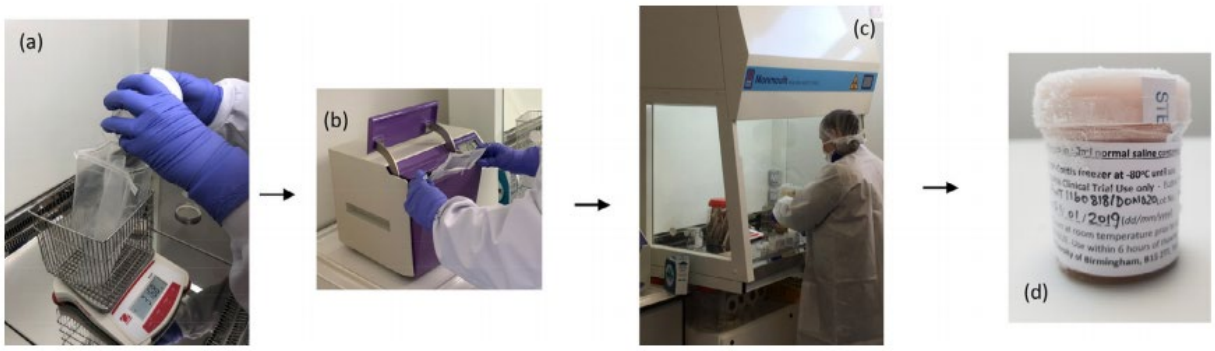


Figure 1 - 5 : Steps involved production of FMT at UoBMTC for STOP-Colitis trial.

(a) Weighing out donor faeces into a Nasco-whirl pak filter bag in a class 2 microbiological safety cabinet. (b) homogenisation of stool using a stomacher. (c) aliquoting of prepared FMT into containers for storage. (d) final packaging and labelling of frozen FMT.

1.2.4 FMT for the treatment of *Clostridium difficile* infection

FMT has proved to be highly successful in treating recurrent and antibiotic refractory CDI. In a meta-analysis of studies up to 2016 (published; lead author), FMT was more effective than vancomycin (RR: 0.23 95%CI 0.07 to 0.80) in resolving recurrent and refractory CDI (122). The clinical resolution / cure rate across all studies was a remarkable 92% (95%CI 89% to 94%) as shown the forest plot in Figure 1 - 6. In interesting trend of increasing response rates were noticed when consecutive courses of FMT were administered following failure of first FMT. Additionally, we found that FMT appeared to be more effective when given by a lower GI compared with upper GI route of delivery with response rates of 95% (95%CI 92% to 97%) versus 88% (95%CI 82% to 94%) respectively (p=0.02). We found that the response rates between fresh and frozen FMT was similar - 92% (95%CI 89% to 95%) versus 93% (95%CI 87% to 97%) respectively (p=0.84). Consequently, FMT for recurrent and refractory CDI is now considered standard practice and is recommended as per the recently published BSG/HIS joint national guidelines.

Unlike IBD, the mechanisms of disease in CDI are better understood where toxins and other putative virulence factors produced by *Clostridiodes difficile* are responsible for the pathogenicity (123). These include shifts in ratio of bile acid compositions, with reductions secondary bile acid concentration that in inhibit toxin production along with changes in specific short chain fatty acids that inhibit growth. Therefore, mechanistic insights into the success of faecal microbiota transplants for CDI have shown restoration of these colonic conditions that are unfavourable to both *C. difficile* germination and toxin production.

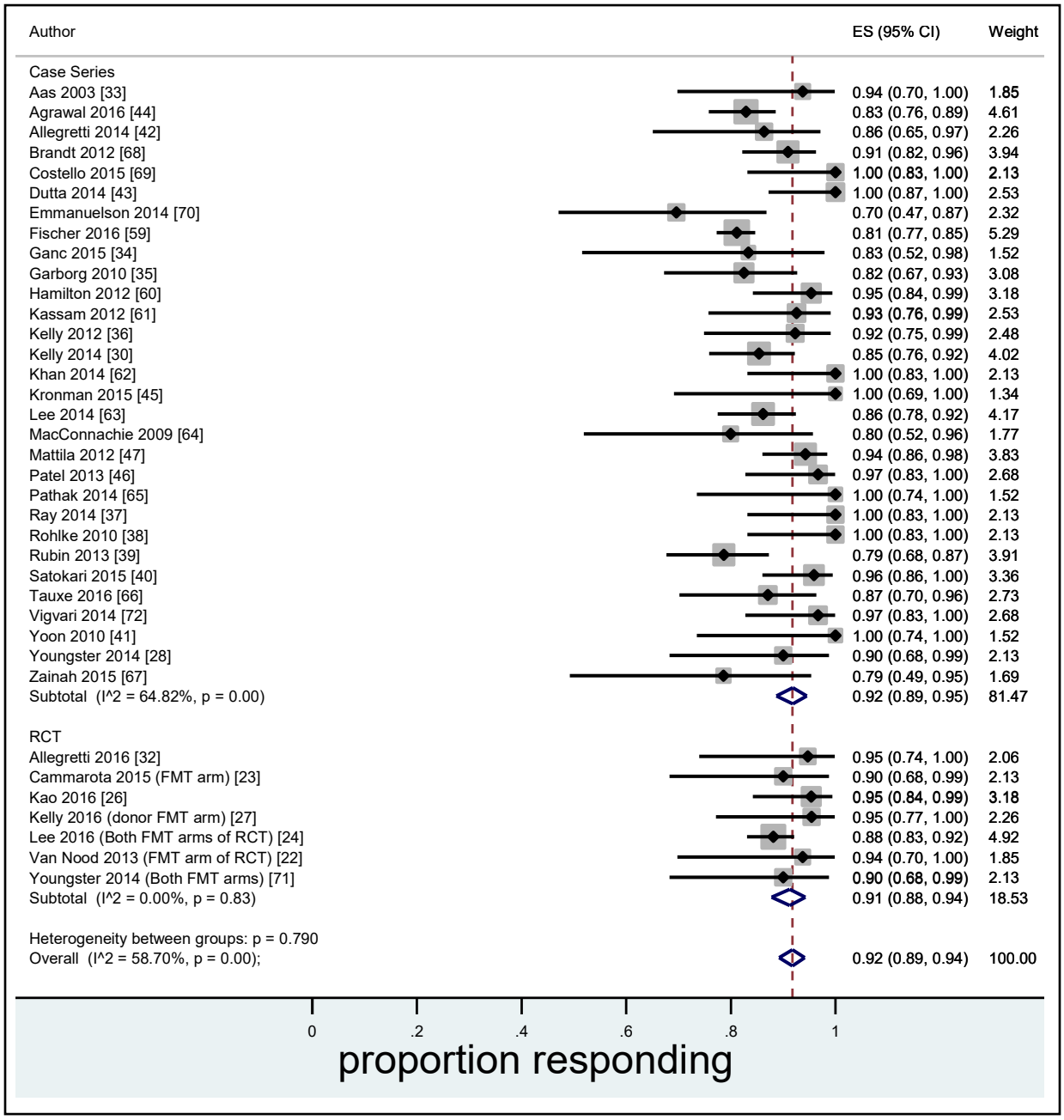


Figure 1 - 6 : Forest plot of studies assessing response to FMT for CDI

The mean pooled overall response for FMT in recurrent and refractory CDI based on all the included 37 studies regardless of the number of infusions was 92% (95% CI 89% to 94%) ES is effect size (95% CI) is the proportion responding with its 95% confidence interval.

Adapted from Quraishi MN et al. Systematic review with meta-analysis: the efficacy of faecal microbiota transplantation for the treatment of recurrent and refractory Clostridium difficile infection. Aliment Pharmacol Ther. 2017 Sep;46(5):479-493.

1.2.5 FMT for treatment of inflammatory bowel disease

The first reported case of FMT for the treatment of IBD was described in 1989 by gastroenterologists Justin Bennet and Mark Brinkman with Dr Bennet achieving sustained remission on treating his own refractory UC with enema delivered stool from a disease-free donor (124). The success of FMT for CDI, coupled with the encouraging signals seen using both antibiotics and probiotics in conjunction with 5ASA compounds in maintaining remission in UC, has led investigators to explore its use in IBD as a target for FMT therapy.

Four randomised controlled trials and a multitude of case reports have been completed to date primarily evaluating the efficacy of FMT in ulcerative colitis. Meta-analysis of the four RCTs demonstrated that clinical remission was achieved in 39 of 140 (28%) patients in the donor FMT groups compared with 13 of 137 (9%) patients in the placebo groups ($p<0.01$) (125). There was marked variability in the designs of each of these four clinical trials ranging from differences in route of administration of FMT (upper GI versus lower GI, fresh versus frozen), total number of FMTs administered (2 to 40 infusions), FMT preparation (anaerobic versus aerobic) and differences in definition of primary outcomes. Consequently, the clinical remission rates varied widely from 24% to 50% in the FMT arm. The trial designs and findings from the studies are summarised in Table 1 - 1.

Table 1 - 1 : Randomised Controlled Trials of FMT in UC

RCT	Moayyedi et al	Rossen et al	Paramsothy et al	Costello et al
Patients (n)	75	48	81	73
Male (n)	44	22	47	40
Age (yrs) FMT vs Placebo	35.8 vs 42.1 (mean)	40 vs 41 (median)	35.6 vs 35.4 (median)	38 vs 35 (mean)
Patients in intervention arm	38	23	40	38
Disease Severity for Inclusion	Mayo score > 4 and	SCCAI ³4 and Mayo endoscopic score of ³1	Mayo score of 4-10 with Mayo endoscopic score ³1 and Physicians global score of £2	Mayo clinical score ≧3 and ≦10 and endoscopic subscore ≧2
Permitted concomitant therapies	Anti-TNF, mesalazine, immunosuppressant (stable dose for 12 weeks). Corticosteroids (stable dose for 4 weeks	Thiopurines, mesalazine, corticosteroids (≤10mg/d) (stable doses for 8 weeks)	Oral 5-aminosalicylates, thiopurines, methotrexate, (stable doses 4 weeks). Corticosteroids ≤20mg/d (stable for 2 weeks but tapered during study over 8 weeks)	Stable dosing of 4 weeks for 5-aminosalicylates, 6 weeks for thiopurines and methotrexate, and 8 weeks for biological agents. Corticosteroids of ≤25 mg, with a mandatory taper of 5 mg per week (failure to wean by week 8 were considered FMT non-responder)
Excluded Therapies	Probiotics or antibiotics within last 30 days	Anti-TNF or methotrexate within 8 weeks and cyclosporine within 4 weeks Probiotics or antibiotics within 6 weeks	Anti-TNF, calcineurin inhibitors within 12 weeks Rectal steroids and 5-aminosalicylate within 2 weeks Probiotics and antibiotics within 4 weeks	Antibiotics or probiotics (minimum duration of withdrawal of use unclear)

Donors	6 anonymous healthy adult volunteers, 1 partner	13 anonymous healthy adult volunteers, 1 partner, 1 friend. 6 recipients had 2 different donors	Blended stool from 3-7 donors (pooled multi-donor) Patients received all infusions from same batch	Mixed unrelated donors (3–4 donors per patient)
Patient Preparation	No bowel lavage	Bowel lavage with 2L macrogol solution, 2L clear fluids	Bowel lavage before colonoscopy	3 L of polyethylene glycol before colonoscopy
FMT preparation	50ml supernatant (50g stool in 300ml water). Administered fresh or stored at -20℃	500ml faecal suspension. Administered fresh within 5 hours of donation	150ml (37.5g blended stool in isotonic saline). Stored at -80℃, then home freezers at -20℃	Colonoscopy 50 g stool in 200 ml saline and glycerol, enema 25 g stool in 100 ml
Placebo preparation	Water	Autologous stool	Saline, colourant, odorant	Autologous stool
FMT Delivery	Retention enema, in left lateral position for 20 minutes	Nasoduodenal tube	1 colonoscopy (terminal ileum or caecum) followed by enemas	Initial colonoscopy (150 ml) then enemas
FMT Frequency	Weekly enemas for 6 weeks	2 times, at weeks 0 and 3	Enemas 5 times a week for 8 weeks	3 (week 0 colonoscopy, 2 enemas week 1)
Follow up	7 weeks	12 weeks	8 weeks	8 weeks
Composite primary endpoint	Remission: Mayo score <3, Mayo endoscopic score 0	Remission: SCCAI ≤2, Mayo endoscopic score reduction of ≥1 point	Remission: steroid-free: total Mayo score ≤2 with Mayo endoscopic score ≤1 and reduction of ≥1 point	Steroid-free total Mayo score ≦ 2 and endoscopic Mayo ≦ 1
Achievement of primary endpoint: FMT vs placebo ITT	9/38 (24%) vs 2/37 (5%) p=0.03	7/23 (30.4%) vs 5/25 (20%) p=0.51	11/41 (27%) vs 3/40 (8%) p=0.021	FMT group: 12/38 (32%), control group: 3/35 (9%) p=0.03

1.3 Immunological mechanisms that are associated with success of FMT in IBD

1.3.1 Insights from clinical studies

A considerable amount of mechanistic work that was incorporated into both the clinical trials and cohort studies primarily focused on changes in the recipient gut microbial and metabolomic profiles and its relationship with clinical outcomes with little consideration of the host biological response. A general theme of an increased α-diversity or richness in the microbiome and a shift of recipient microbial profiles towards those of donors were observed with some studies suggesting colonisation by specific donor-derived taxa was associated with clinical benefit (126). However, immunological consequences of FMT in IBD have been very poorly described.

Immunophenotyping of lamina propria mononuclear cells (LPMC) and peripheral blood mononuclear cells (PBMC) in patients recruited in the RCT conducted by Costello and colleagues was described in supplementary data (127). They failed to find any significant change in proportions of γδ T cells, NK cells and T cells (including subsets: Memory, CD4, CD8 or Tregs) in LPMCs as a result of FMT. Interestingly on analysis of PBMCs however they found a slight increase in gut homing CD4 T cells (defined as CD4+ CD45RO+ β7+) cells following FMT which was only just significant ($p=0.05$) when adjusted for clinical disease activity scores. The gut homing T regulatory cells subset in PBMCs did not however change post FMT. A detailed methodology used for cell isolation and immunophenotyping (including representative gating strategy) was not described. It also was not clear whether any shifts in immune subsets were seen in those that responded to FMT compared to those that did not. Analysis of immunological changes was not explored in any of the other three published randomized controlled trials.

There are over 40 case series exploring the efficacy of FMT in IBD of which only a handful have reported immunology outcomes. A pilot study of 19 patients with active Crohn's disease demonstrated an increase in proportion of colonic mucosal Tregs (defined as CD4+CD25+CD127lo) 12 weeks after a single colonic infusion of FMT (128). The change was not different in responders compared to non-responders (mean 5% versus 5.3% respectively) or in a Th17 like cell population (CD4+CD39+CD161+). A study of 19 patients with moderate to severe UC failed to show a change in a large panel of serum cytokines including IL-10 and IL-17 following a single upper GI infusion of FMT regardless of clinical response (129). An open label pilot of single FMT delivery by colonoscopy in 20 patients active UC revealed a decrease in colonic mucosal Th1 and Treg cells with no difference in Th17 cell population post FMT(130). Seven patients in this study achieved clinical remission however a subgroup analysis of mucosal immunophenotype in these responders was not presented. A case series that failed to show a beneficial clinical response in eight patients with chronic pouchitis after a single intra-gastric dose of FMT also, perhaps not surprisingly, failed to demonstrate any change in lamina propria dendritic cell phenotype and cytokine profiles (131). A seminal study that incorporated an open-label pilot of FMT explored the role of bacteriophages in altering mucosal immune responses. In this study, 20 patients with active UC treated with a single colonoscopic infusion of FMT showed a positive correlation between CD4+ T cell production of IFNγ from rectal mucosal biopsies and the relative abundance of total gut viral reads specifically the bacteriophage *Caudiovirales* (132). They found that this bacteriophage was significantly enriched in patients who failed to respond to FMT. Mechanistically they demonstrated that introduction of *Lactobacillus*, *Escherichia*, and *Bacteroides* bacteriophages into germ free mice lead to immune cell expansion and stimulated IFNγ via the nucleotide-sensing receptor TLR9 in the gut.

Immune checkpoint inhibitor (ICI) targeting cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1), and programmed cell death ligand 1

(PD-L1) has been shown to improve survival across numerous cancer types by increasing T-cell activation and driving an effective antitumor immune. A common adverse reaction associated with ICI is colitis that can be quite severe and resemble that seen in IBD. A case report of two patients with steroid and biologic refractory ICI associated colitis demonstrated a successful response with FMT (133). These patients had an increased abundance of pathogenic Gammaproteobacteria and notable absence of potentially protective Bacteroidia. This immunological response after FMT was associated with a substantial reduction in the colonic mucosal CD8+ T-cell density and increase in FoxP3+ CD4 cells. There was a concomitant expansion in the population of *Clostridia* and *Blautia*. There was also an increase in *Bifidobacterium* species following FMT and this was recently reported to abrogate ICI-related toxicity in a murine model (134). In this study, mice with DSS induced colitis and anti–CTLA-4 blockade, treatment with *Bifidobacterium* ameliorated colitis. This protective effect was abrogated in Treg depleted mice. Collectively these findings indicate an emerging role of FMT and specific agents in the gut microbiota in mitigating inflammation via induction or modulation of regulatory T cells function.

It is clear that immunological outcomes have been poorly explored in clinical FMT studies in IBD with studies focusing primarily on gut microbial analysis. Nevertheless, these analyses have provided valuable indirect insights into microbial interactions that potentially modulate immune mediated inflammatory responses. Gut microbiota profiles in FMT responders consistently show a significant shift towards butyrate producing species of bacteria that are known to induce Tregs and promote IL-10 production (102, 103). Rossen and colleagues reported that at 12 weeks of follow up, the microbiota of responders in the FMT group was similar to that of their healthy donors and remission was associated with proportions of *Clostridium* clusters IV and XIVa (135). The FOCUS trial conducted by Paramsothy and colleagues reported that increased abundances of species belonging to *Clostridium* cluster XIVa and XVIII such as *Roseburia intestinales* were associated with positive outcomes

following FMT (136, 137). Furthermore members of *Clostridium* clusters IV and XIVa in the *Ruminococcaceae* and *Lachnospiraceae* family were significantly enriched in stool of a donor (donor B) who was associated with the highest rate of response following FMT in recipients in the in the study conducted by Moayyedi and colleagues (138). In the analysis of mechanistic outcomes from this study, a trend was seen for responders having microbiota that was more similar to donor B than non-responders.

1.3.2 Insights from animal studies

Immune responses to FMT have been explored in a few studies using IBD and non-IBD mouse models. Functional effects of therapeutic FMT administration during experimental colitis on innate and adaptive immune responses in the intestinal mucosa were explored in a pivotal study (139). Mucus and faeces derived from normobiotic mice were gavaged into CXCR6-$^{EGFP/+}$ reporter mice (for fluorescent T cell tracking) with DSS-induced colitis resulting in reduction of intestinal inflammation and histological inflammation scores compared to control DSS colitis mice. FMT-treated DSS-colitis mice demonstrated higher amounts of colonic IL-10 as well as increased frequencies of IL-10-producing CD4+ T cells and invariant natural killer T cells (iNKT) in comparison to control DSS-treated mice. FMT treatment was also associated with a reduction of macrophages and neutrophils along with a non-significant increase in the frequency of Foxp3 + Treg cells. Intriguingly this increase in IL-10 secretion by T cells normalised upon resolution of inflammation. Pharmacological blockade of IL-10 receptor hampered protective effects of FMT suggesting a direct contribution of the microflora in the IL-10-mediated control of inflammation. Furthermore, there was a decrease in innate lymphocytes ILC2 and ILC3, macrophages and neutrophils in the lamina propria in FMT treated DSS-colitis mice. FMT induced a significant reduction in the number and level of expression of colonic MHC-II-expressing APC (including dendritic cells and macrophages). LPMC exposed to FMT-derived microbiota showed reduced levels of pro-

inflammatory cytokines, such as TNF-α, IL-1β and IFN-γ. Camp and S100A8, two antimicrobial peptides playing anti-inflammatory roles during acute intestinal inflammation were upregulated upon FMT administration. Gut microbiota analysis of FMT treated mice showed significant increases of commensals including species belonging to *Lactobacillaceae* and *Streptococcus* along with of the SCFA-producing taxa *Erysipelotrichaceae* and *Ruminococcaceae*. This instrumental work demonstrated the beneficial anti-inflammatory effect mediated by FMT in modifying immune cell frequencies, reduction of pro-inflammatory colonic IFN-γ and IL-1β, increase in specific antimicrobial peptides and mucins, and a decrease of MHC-II antigen presentation by APC. Crucially FMT induced a shift toward a tolerogenic IL-10 secreting cytokine profile that ameliorated intestinal inflammation.

In a separate study FMT was shown to upregulate the expression of aryl hydrocarbon receptor (AhR), IL-10, and TGF-β in colon tissues in mice with DSS-induced colitis and was associated with improvement of severity of colon mucosa injury and histological parameters (140). This correlated with gut microbial recovery of *Lactobacillus* and *Bifidobacterium* species and an increase in tryptophan levels, which, as demonstrated by this study, results in differentiation of immune cells in order to promote or regulate the release of anti-inflammatory factors (141-143).

Administration of FMT to mice following perturbation of gut microbiota with 8 weeks of broad spectrum antibiotics led to re-establishment of small intestinal CD4+, FoxP3+ CD8+ and B220+ as well as of colonic CD4+ and FoxP3+ cell numbers as early as 7 days post-FMT (144). Antibiotic treatment resulted in reduced cytokine production (IFN-γ, IL-17, IL-22, and IL-10) by CD4+ T cells. These effects were, however, completely restored following FMT. Seven days post-FMT, a strong IL-10 response was observed in the colon, which at 28 days

reached levels similar to antibiotic untreated control mice. A follow up study showed introduction of gut commensals *Escherichia coli* and *Lactobacillus johnsonii* increased the frequencies of Tregs, activated dendritic cells and intestinal memory/effector T cell populations 28 days after antibiotic-induced microbiota depletion (145). This effect was inferior to that seen with FMT and only *Lactobacillus johnsonii* was able maintain colonic IL-10 production. In another antibiotic induced dysbiosis model of BALB/c mice, intragastric FMT resulted in earlier reductions of α-defensins 5 and 6 along with an increase in β-defensin 2 and concentration of secretory IgA in comparison to those that recovered spontaneously (146).

Administration of FMT following ileocolic resection in an IL10 -/- murine model prevented ileal inflammation but worsened colitis and was associated with increases in colonic mucosal TNF-α, IFN-γ and IL-2 levels compared to non-operative controls. These paradoxical findings are interesting and support the IL-10 dependent regulatory immune mediated mechanisms of FMT that appear to be abrogated in an IL-10 knockout model of IBD (147). A study with antiretroviral-treated, chronically simian immunodeficiency virus (SIV) infected rhesus macaques that received antibiotics showed significant increases in the number of peripheral Th17 and Th22 cells following administration of FMT. Reduced CD4+ T cell activation (based on expression of HLA-DR) in gastrointestinal tissues was also observed and this correlated negatively with abundance of butyrate producer *Roseburia* (148).

1.3.3 Summary of findings

The evidence presented so far outlines key themes explored to date around the immunoregulatory mechanisms of FMT in IBD and this summarised in Figure 1 - 7. Administration of FMT is associated with increase in production of specific antimicrobial

peptides, secretory IgA and mucin thereby minimising pathogen invasion by antigen/pathogen dependent and independent targeting. There is some evidence to suggest a reduction of neutrophils, macrophages, pro-inflammatory cytokines and downregulation of MHC-II dependent presentation of bacterial antigens in response to FMT administration. However collectively the strongest evidence primarily from animal models and clinical studies indicate its significance in the induction of colonic mucosal Tregs with an IL-10 dependent resolution of inflammation in IBD. This amelioration of colonic inflammation was associated with enrichment of specific *Clostridium* clusters that include the SCFA producing families *Ruminococcaceae* and *Lachnospiraceae* and genus *Roseburia*.

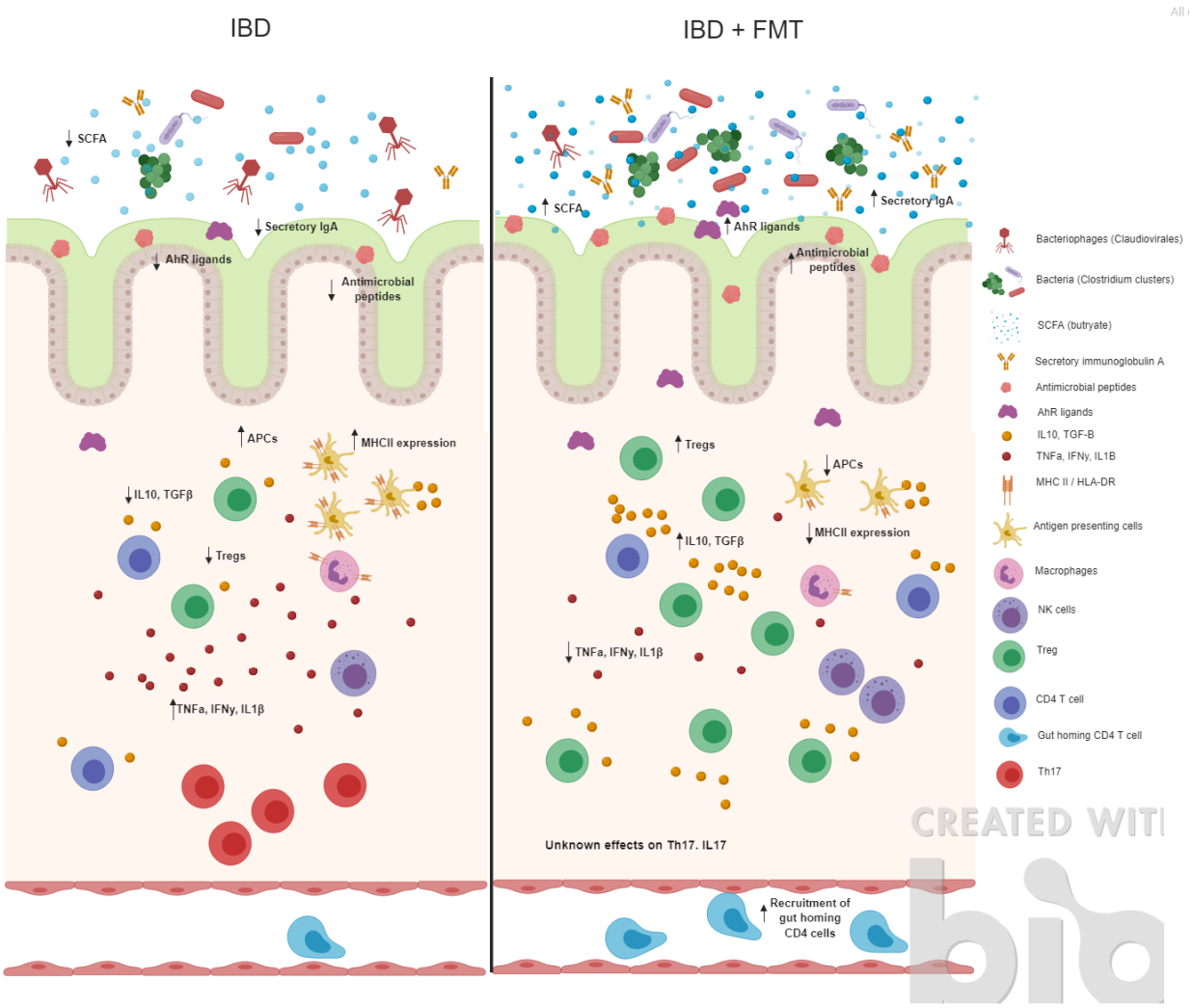


Figure 1 - 7 : Summary of evidence to date demonstrating intestinal mucosal immunological changes associated with FMT

FMT is associated with an increase in production of antimicrobial peptides including cathelicidin antimicrobial peptide (Camp), S100A8, specific defensins, secretory immunoglobulin A and mucin. A reduction of antigen presenting cells including neutrophils and macrophages and upregulation of Tregs, IL-10 secreting CD4 T cells and circulating gut homing CD4 T cells following FMT. Consequently, there is an increase in IL-10 and TGFβ production and a reduction of pro-inflammatory cytokines including TNF-α, IFN--γ and IL-1β. A downregulation of MHC-II dependent presentation of bacterial antigens via dendritic cells is also noted following administration of FMT. These findings are associated with amelioration of intestinal inflammation. Administration of FMT is associated with enrichment of specific *Clostridium* clusters that include the SCFA producing families *Ruminococcaceae* and *Lachnospiraceae* and genus *Roseburia* in clinical studies.

1.4 Conclusions

Cumulating evidence supports the causal contribution and centrality of an interaction of a disrupted gut microbiota with a primed immune response in setting up a proinflammatory environment that leads to the development and progression of IBD. Modulation of this dysbiosis by FMT shows promise in the treatment of IBD, however, strong data regarding the biological basis of this success is lacking. Insights from clinical studies and animal experiments suggests both association and direct contribution of changes in specific members of the gut microbial community as a consequence of FMT that dictate a shift in homeostatic balance towards intestinal immunoregulatory pathways. Further careful mechanistic exploration from human FMT studies with the aim to explore important interactions between the microbiome and the immune system will uncover potential targets for intervention and, equally importantly, the microbial triggers for IBD.

1.5.1 Host microbiota interactions governing primary sclerosing cholangitis associated inflammatory bowel disease

Primary sclerosing cholangitis (PSC) is a rare, chronic, cholestatic liver disease that is characterised by inflammation and fibrosis of intra and extrahepatic bile ducts. This results in formation of multifocal strictures and progressive fibrosis of bile ducts that eventually leads to cirrhosis of the liver and liver failure. Hepatic fibrosis is promoted buy these biliary changes, ultimately leading to liver cirrhosis and liver failure. PSC occurs more commonly in men than in women at a ratio of 2:1 and the mean age at the time of diagnosis is approximately 40 years. The prevalence of PSC appears to be considerably higher in the Western World compared to developing countries however, this is likely to change in congruence with the increasing epidemic of IBD seen in Asia and Middle East. In the absence of any disease or prognosis modifying pharmacological therapy, liver transplantation currently represents the only curative option. Without liver transplantation the median life expectancy after diagnosis of PSC is only 13.2 to 21.3 years.

PSC is the commonest hepatobiliary manifestation of IBD (PSC-IBD) and is present in approximately 70-80% of PSC patients. Around 85-90% patients PSC with concurrent IBD (PSC-IBD) have ulcerative colitis (UC) and the remaining have Crohn's colitis or Crohn's ileocolitis. The phenotype of colonic inflammation occurring in PSC is unique and differs from the typical phenotype seen in patients with conventional IBD. Patients with PSC-IBD typically have relatively mild intestinal disease activity with a higher incidence of extensive colitis that typically manifests as pancolitis, with rectal sparing and backwash ileitis. Importantly, the risk of colorectal cancer is 4 to 5 times higher in the setting of PSC and IBD versus IBD alone, but 10-fold higher compared with the general population. The risk of PSC and UC is elevated in first-degree relatives of patients with PSC and large-scale genome wide association studies have identified multiple shared and non-shared genetic loci that underlies this risk.

Uncovering the aetiopathogenesis of primary sclerosing cholangitis (PSC) is one of the major challenges we currently face in hepatology. The pathogenesis of PSC is poorly understood and is thought to be multifactorial with an interplay between immune dysregulation, gut microbial and bile acid dysregulation in a genetically pre-disposed individual.

Genome-wide association studies (GWAS) and arrays have identified numerous genes linked to the development of PSC-IBD. These large-scale case–control studies have discovered over 20 risk loci, with most notably a remarkable human leukocyte antigen (HLA) association that mirrors other autoimmune diseases (149, 150). The translation of these discoveries is challenging as these regions of genes do not confer independent causal effects as none of these point toward an obvious biological process. PSC-IBD appears to be clearly genetically distinct to IBD as less than half of the risk loci overlap between the two diseases with associations primarily stronger in PSC-IBD than IBD. As IBD is reported in up to 80% of cases of PSC, this limited genetic risk loci overlap, and distinct clinical phenotype possibly suggests that the mechanisms that drive PSC-IBD are unique and possibly represents an independent disease (151). These host genetic differences in PSC-IBD may however influence other key factors such as the composition of gut microbiota to promote a dysregulated immune response. For example, variants of the suspected risk gene FUT2 are associated with dysbiosis and are known to alter the biliary tract microbiota in PSC-IBD (152). Furthermore, the robust HLA association suggests disease-specific antigens, possibly derived from the gut microbiota may be responsible for driving pathogenesis in PSC-IBD. T-cell receptor (TCR) sequencing analysis of the T cells infiltrating PSC-IBD explanted livers has uncovered evidence of antigen-driven clonal expansion (153). Moreover, memory T cells of common clonal origin are found at a significantly increased frequencies in paired gut and

liver samples of patients with PSC-IBD compared to controls implying gut derived antigenic triggers initiating or perpetuating immune activation along the gut-liver axis (154, 155).

Although ulcerative colitis (UC) occurs in up to 80% of patients with PSC (PSC-IBD) there are crucial differences in clinical manifestation of colonic inflammation and clear distinctions amongst genetic polymorphisms identified through genome wide association studies.[1] A substantial component of the genetic architecture of PSC is, however, not shared with UC and genetic correlation modelling would generate a UC comorbidity rate of only 1.6% in patients with PSC. This tends to suggest that the observed extent of comorbidity and causality between PSC and UC cannot be fully explained by shared additive genetic effects of common variants. It is almost certain that other factors must play a role, such as the gut microbiota and other environmental factors not captured through GWAS studies and imputation data. These phenotypic and genotypic differences have led to the proposal that colonic inflammation in the context of PSC-UC is aetiologically and pathophysiologically a different to that seen in UC.

1.5.2 Gut microbiota in PSC-IBD

In view of the growing evidence to support the causal role of dysbiosis in IBD, the close association of PSC with IBD raising the suspicion that gut microbiota might also play a role in the pathogenesis of PSC. In support of this supposition, several recent studies have shown that patients with PSC and PSC-IBD have a distinct gut microbiota compared to IBD patients and healthy controls as summarised in Table 1 - 2.

Sabino et al demonstrated that the faecal microbiota in patients with PSC is characterised by decreased microbial diversity, together with an increased abundance of Enterococcus, Fusobacterium and Lactobacillus (156). Similar dysbiosis was present in patients with both PSC and PSC-IBD and was distinct from IBD and independent of treatment with ursodeoxycholic acid. An increased abundance of Enterococcus was associated with increased levels of serum alkaline phosphatase. Similar findings were reported by Kummen et al, who found that the abundance of the genus Veillonella was markedly increased in PSC compared to healthy controls and patients with ulcerative colitis (157). In work that I had previously conducted study, we found a distinctive population of mucosa-adherent bacteria in patients with PSC-IBD compared to IBD and healthy controls (158). These microbial profiles showed significant increases in Escherichia, Lachnospiraceae and Megasphera along with a reduced abundance of Prevotella and Roseburia and a near-absence of Bacteroides. Yet another study observed a significant increase in Barnesiellaceae and shifts in the abundance of Clostridiales and Bacteroidales in intestinal mucosal biopsies from patients with PSC.

Table 1 - 2 : Studies of gut microbiota in PSC

Study	Cohort studied	Tissue sampled	Key findings
Kummen M et al. Gut 2016	85 with PSC, 36 with UC and 263 healthy controls	Stool samples	Significantly distinct microbiota in PSC compared to UC and healthy controls. PSC and PSC-UC microbiota was similar. Veillonella genus was significantly increased compared to the other groups.
Torres J et al. Aliment Pharm Ther 2016	20 with PSC (19 with PSC-IBD and one with PSC-only), 15 with IBD-only and nine healthy controls	Pan-colonic and terminal ileal biopsies	No differences in the global microbiome profile, however increased abundance of Blautia and Barnesiellaceae in PSC group along with major shifts in Clostridiales.
Kevans D et al. J Crohn's Colitis 2016	31 with PSC-UC and 56 with UC	Left sided colonic biopsies	No strong PSC-specific microbial associations in UC identified.
Rossen NG et al. J Crohn's Colitis 2015	12 with PSC, 11 with UC, and 9 controls	Ileocecal biopsies	No cluster differentiation of microbiota between the groups. Reduced amounts of uncultured Clostridiales II in PSC biopsies in comparison with UC and healthy controls.
Quraishi MN et al. Gut 2016	11 with PSC-IBD, 10 with IBD and 9 healthy controls	Pan-colonic biopsy	PSC-IBD microbiota significantly distinct from the other groups. Significant increase in Escherichia, Lachnospiraceae and Megasphera and decrease of Prevotella and Roseburia and a near-absence of Bacteroides in PSC-IBD.
Sabino et al. Gut. 2016	52 with PSC, 52 healthy controls, 13 UC and 30 with Crohn's disease	Stool samples	Microbiota of patients with PSC was characterised by decreased microbiota diversity, and a significant overrepresentation of Enterococcus (p=3.76e-05), Fusobacterium (p=3.76e-05) and Lactobacillus (p=0.0002) genera.
Rühlemann et al. Ailm Pham Ther. 2019	137 patients with PSC (n = 75 with colitis), 118 with UC and 133 healthy controls	Stool samples (unclear if this was a reanalysis)	Reduced diversity with an increase in Proteobacteria and Parabacteroides. No difference between PSC and PSC-IBD
Bajer et al. World J Gastro 2017	43 PSC patients with (n = 32) or without (n = 11) IBD, 32 UC patients, and 31 healthy controls	Stool samples	Increase in Rothia, Enterococcus, Streptococcus, Veillonella and decrease in Adlercreutzia equolifaciens and Prevotella copri
Lemoinne S et al. Gut. 2019	PSC and concomitant IBD (n=27), patients with PSC only(n=22), patients with IBD (n=33) and healthy subjects (n=30)	Stool samples	Fungal dysbiosis - Increase in exophiala and reduction in S. cerevisiae Bacterial dysbiosis - reduction in Firmicutes↓ along with increase in Veillonella and Proteobacteria

Recently Rühlemann et al published the largest study to date in this field consisting of 388 individuals (137 with PSC) based in Norway and Germany (159). They confirm that the gut (faecal) microbial profiles of patients' PSC were distinct to UC and healthy controls and this was independent of medication or presence of colitis; albeit there was a higher median faecal calprotectin level in the UC cohort. Intriguingly they found no differences in the gut microbiota between PSC and PSC-IBD proposing that this dysbiosis seen in PSC was independent of colitis. Through a meta-analysis, they identified both known and novel differences in taxa in PSC that may potentially have a pathophysiological significance such as increases in the proinflammatory lipopolysaccharide containing Gammaproteobacteria.

1.3.3 Exploring casual links with gut microbiota in PSC pathogenesis

There is accumulating evidence that demonstrates PSC is associated with differences in gut microbiota. In order to understand what this actually means we now need to move towards carefully conducted studies that explore causation. Genetic association studies in PSC have identified genetic risk variants in FUT-2 gene, which is implicated in the handling of translocated gut bacteria and antigens associated with PSC and IBD (152). Murine models have shown that compromised mucosal integrity in an inflamed gut results in biliary disease and impaired epithelial barrier function is associated with biliary infection. Interestingly, in a germ-free murine model of PSC (multidrug-resistance-2 knockout mice), secondary bile acids were absent, and the mice showed biochemical and histological features of PSC (160).

A consistent finding of gut microbiota studies in PSC is the increase in abundance of the genus Veillonella in patients with PSC. This bacterium contains genes for copper amine oxidase proteins, which may influence expression of vascular adhesion protein-1 (VAP-1), an adhesion molecule implicated in aberrant trafficking of gut-homing lymphocytes to the

liver in patients with PSC (161). In addition, bacteria that produce biogenic amines or possess amine oxidase activity show an increased abundance in PSC patients and may contribute, via their metabolites, to pathogenesis of disease (158). Furthermore, there appears to be enrichment of bile salt hydrolase expressing bacteria such Lactobacillales in PSC compared to controls. I performed a post hoc inferred functional composition analysis of my previously published data exploring mucosally adherent gut microbial in PSC-IBD. Through this, I demonstrated specific changes in potential gut microbial function in particular those associated with bile acid metabolism through various mechanisms including PPAR signalling pathways (Figure 1 - 8).

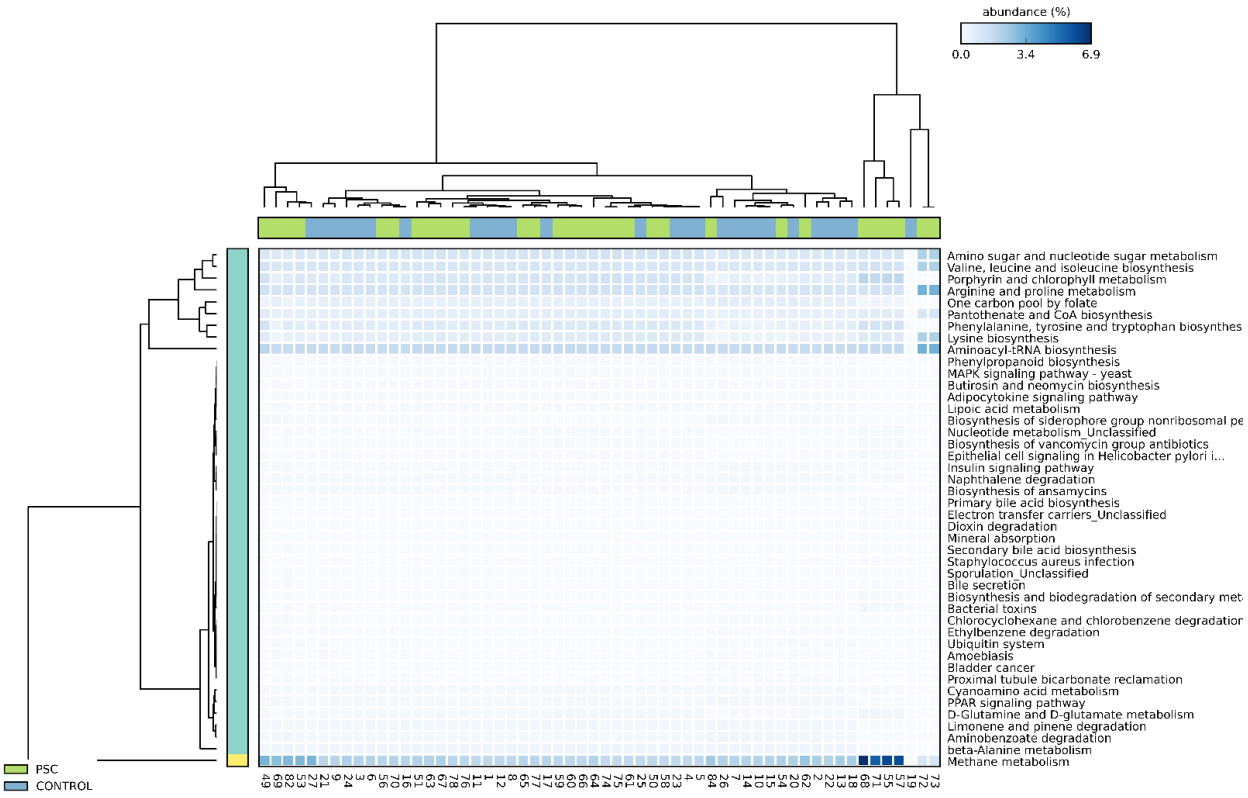


Figure 1 - 8 : Inferred gut microbial functional analysis of PSC-IBD vs healthy controls

Key mechanisms of potential gut microbial function appear to be upregulated in PSC-IBD compared to healthy controls as determined by metagenomic functional inference of 16S rRNA datasets. Pathways including bile acid synthesis, bile secretion, PPAR synthesis and epithelial cell signalling are upregulated in PSC-IBD suggesting potential disease mechanisms influenced by the altered gut microbiota in PSC-IBD.

1.3.4 Insights through murine models in PSC / PSC-IBD

Numerous animal models have been developed to study PSC, however given the ambiguities regarding the aetiopathogenesis of PSC, it is unsurprising that no single model has fully recapitulated its biochemical, biliary, colonic (PSC-IBD), and premalignant features (162). The most widely studied MDR2 (ABCB4) knockout murine model which exhibits only a biliary phenotype of PSC and the primary mechanism of liver injury is not truly representative of PSC (163). Other murine models include the experimental biliary obstruction C57BL/6J mice, lithocholic acid induced cholangitis and models involving biliary epithelial and endothelial cellular injury (164, 165). Ovalbumin (OVA)-BIL mice drive aberrant expression of a membrane form of ovalbumin on biliary epithelium (166). Adoptive transfer of OVA specific CD4+ and CD8+ T cells has been shown to result in dose dependant biliary centred inflammation and necrosis similar to that seen in PSC. These mice do not however develop a colonic phenotype (167). An immunological model of PSC that has the closest recapitulation of the PSC and PSC-IBD phenotype is IL2RA −/− knockout mice. The IL-2 cytokine has essential functioning role in immunological tolerances and immunity through direct effects on CD4 and CD8 T cells. IL2RA encodes the alpha chain of the IL-2 receptor and binds to the IL-2 cytokine in order to mediate its signalling effects (168). GWAS studies have identified IL2RA as being a key susceptibility risk locus that overlap between PSC and UC (169). IL2-/- mice spontaneously develop intestinal and biliary inflammation (170). PSC-associated variants within IL2RA lead to reduced expression of IL2RA, which have implications for induction of immune tolerance possibly against gut commensals (171).

1.3.5 Insights through studies exploring use of oral vancomycin in PSC / PSC-IBD

The strongest causal evidence to date comes from studies exploring treatment of PSC with oral vancomycin. To date there have been two RCTs comparing the efficacy of antibiotics (vancomycin and metronidazole) against placebo in PSC patients, while one non placebo-controlled RCT compared the effectiveness of low and high doses of vancomycin and metronidazole in PSC patients. A recent systematic review and meta-analysis found that antibiotic therapy and in particular, vancomycin was associated with a statistically significant reduction in alkaline phosphatase (ALP), PSC Mayo Risk Score (MRS) and total serum bilirubin level by 33.2, 36.1, and 28.8%, respectively. The clinical and biochemical response was greatest for the non-absorbable antibiotic vancomycin as compared to the other antibiotics, which highlights the potential role for gut microbiota in the aetiopathogenesis and treatment of PSC. More recently, a single centre retrospective review of patients with PSC-UC with active colitis demonstrated clinical and endoscopic response / remission in all the eight patients (100%) treated with an 8-week course of oral vancomycin.

In addition to likely direct immunomodulatory mechanisms, vancomycin specifically targets Gram positive bacteria. Specific members of these bacteria are potentially involved with the dehydroxylation of primary bile acids that are produced by the liver, into secondary bile acids present in the distal small bowel and colon. As a proposed leading hypothesis for colonic inflammation in PSC is thought to be secondary to colonic mucosal bile acid toxicity, this may be a potentially significant mechanism by which vancomycin improves both colonic inflammation and liver biochemistry in this context. Equally importantly, it highlights the role gut microbiota – bile acid – mucosal axis in the development and progression of PSC-IBD. However, no study to date has explore gut microbial changes associated with treatment with vancomycin for PSC / PSC-IBD.

Gut microbiota modulation in PSC has also been explored with the therapeutic use of FMT. In a preliminary open-label pilot study consisting of ten patients treated with a single FMT by colonoscopy revealed that 30% (3/10) had a ≥50% decrease in ALP levels with an increase in alpha diversity post FMT. There are currently several large-scale trials ongoing worldwide exploring FMT for treatment of PSC-IBD.

1.3.6 Conclusion

There is accumulating evidence that demonstrates PSC is associated with differences in gut microbiota. In order to understand what this actually means we now need to move towards carefully conducted studies that explore causation. Very early exploratory clinical trials investigating modulation of gut microbiota with vancomycin and faecal microbiota transplantation in PSC have shown possible potential.[8]–[10] Harnessing such studies for mechanistic exploration of changes in host pathophysiology in relation to shifts in gut microbiota may hold a key to understanding and treating this complex disease better (Figure 1 - 9).

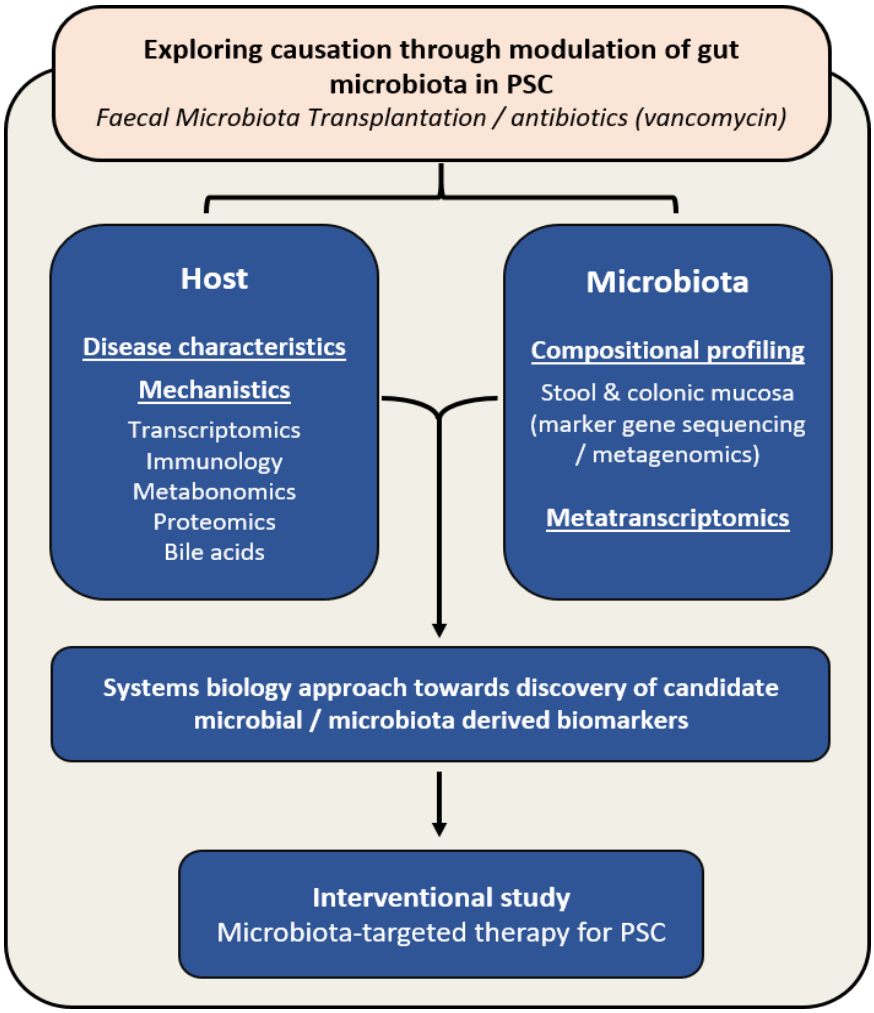


Figure 1 - 9 : Future directions towards exploring microbiota causation in PSC

Association studies have consistently revealed dysbiosis in PSC-IBD; however, its significance in disease pathogenesis remains unclear. In order to determine causal roles and explore novel therapeutic avenues, we now need to move towards carefully conducted studies that adopt a systems biology approach in investigating host - microbiota interactions in PSC-IBD. These approaches should include cross sectional analysis of deep multi-omic integration studies as well as microbiota modulation studies with FMT and antibiotic therapies.

*Adapted from Quraishi MN and Shaheen WA. Editorial: gut microbial profile associated with primary sclerosing cholangitis—what is new and how do we progress from here? Aliment Pharmacol Ther. 2019 Sep;*50(5):605-606

Parts of this chapter have now been published in the following articles:

1. **Quraishi MN**, Shaheen WA. Editorial: Gut microbial profile associated with primary sclerosing cholangitis – what is new and how do we progress from here? **Aliment Pharmacol Ther**. 2019
2. **Quraishi MN,** Shaheen WA, Oo Ye, Iqbal T. Immunological mechanisms underpinning faecal microbiota transplantation for the treatment of inflammatory bowel disease. **Clin Exp Immunol.** 2020 Jan;199(1):24-38. doi: 10.1111/cei.13397. Epub 2019 Nov 27.
3. **Quraishi MN**, McNally A, van Schaik W. Do we really understand how faecal microbiota transplantation works? **EBioMedicine**. 2019 Mar 15. pii: S2352-3964(19)30164-1
4. **Quraishi MN**, Critchlow T, Bhala N, Sharma N, Iqbal T. Faecal transplantation for IBD management-pitfalls and promises. Br Med Bull. 2017 Dec 1;124(1):181-190.

1.4.1 Thesis Hypothesis and Aims:

Hypothesis: Gut microbiota drive pathogenic mechanisms of colonic inflammation in UC and PSC-IBD through multiple and potentially modifiable host biological pathways

Alterations in gut microbiota are a cardinal feature of both inflammatory bowel disease (IBD) and primary sclerosing cholangitis associated inflammatory bowel disease (PSC-IBD). There is now increasing evidence to suggest that this dysbiosis is likely to play a causal role in the development of IBD and possibly PSC-IBD. The mechanisms by which these aberrant gut microbiota interact with the host to cause colonic inflammation is however, less clear in IBD and more so, in PSC-IBD. This thesis has two primary aims:

1) Investigate mechanisms by which gut microbiota play a causal role in the pathogenesis of ulcerative colitis (UC), with a particular focus on immunological pathways. I intend to do this through several indirect and direct approaches.
   a. Identify if there are inherent intra-individual differences in gut microbiota at inflamed and non-inflamed segments of the bowel in patients with UC.
   b. Phenotype the colonic immune response in UC
   c. Investigate clinical, immunological and transcriptomic effects of modulation of gut microbiota through faecal microbiota transplantation in patients with UC.

2) Characterise host - gut microbial pathways associated with colonic disease in PSC-IBD. In order to achieve this, I will apply a comparative systems biology approach capturing the colonic mucosal transcriptome, mucosal immunophenotype and mucosally adherent gut microbiota profiles in patients with PSC-IBD, UC alone and healthy controls.

CHAPTER 2

Materials and Methods

Methods

This section covers relevant methodology for wet and dry lab analysis undertaken for the thesis. Clinical trial protocol for Chapter 5 is described separately in that chapter.

2.1 Clinical

2.1.1 Study population for exploratory analysis - *(Chapter 3, Chapter 4, Chapter 6)*

Patients with ulcerative colitis (UC), primary sclerosing cholangitis with concurrent inflammatory bowel disease (PSC-IBD), and healthy controls (HC) were recruited from outpatient clinics and endoscopy lists for this study. PSC-IBD and UC were documented according to standard of care and in keeping with European guidelines on diagnosis (EASL and ECCO) (172, 173). Healthy controls were individuals with no known co-morbidities having had a normal colonoscopy (other than haemorrhoids) as part of investigation for rectal bleeding. Subjects were excluded if they had a history of antibiotic and/or probiotic use in the last three months. This exclusion criteria is not based on strong evidence as different antibiotics result in different rates of recovery of the gut microbiome. However, a three month exclusion is part of accepted standardised protocols in observational studies and clinical trials. Only patients with large duct PSC (radiological with or without histological confirmation) were recruited as part of the PSC-IBD cohort. As all patients in this study had UC or UC phenotype with PSC-IBD, disease activity was assessed by Mayo scoring (with endoscopic Mayo subscores). Further details are provided within the individual chapter methodology sections.

2.1.2 Ethical approval

Ethical approval for colon biopsy collection and analysis was given by the University of Birmingham Human Biomaterials Resource Centre (HTA Licence 12358) as part of the HBRC application 13-145. Approval for collection of whole blood for peripheral blood mononuclear cell immunophenotyping was obtained through Yorkshire & The Humber - Bradford Leeds Research Ethics Committee' – REC reference: 16/YH/0100

2.1.3 Sample collection

Up to five pairs of colonic mucosal biopsies were taken from the sigmoid colon. Whilst accepting that patients with PSC-IBD may have dominant right sided colonic inflammation, sigmoid colon biopsies allowed standardisation for sample collection between sigmoidoscopy and colonoscopy and controlled for differences in extent of inflammation (for example left sided versus pancolitis with both patient having at least inflammation in the sigmoid). Two pairs were immediately collected in Qiagen RNAlater Tissue Protect tubes for mucosal host transcriptomic and mucosally adherent microbiota analysis. The tubes were incubated on ice for 4 hours and then stored at -80C. Three biopsy pairs were collected in Miltenyi Biotec gentleMACS C Tubes containing complete RPMI (RPMI-1640 medium with L-glutamine supplemented with 10% Fetal Bovine Serum and Penicillin / Streptomycin / Glutamine) for mucosal immunophenotyping. Demographics and clinical data were noted at the time of sample acquisition.

2.2 Microbiota analysis - mucosally adherent 16S rRNA profiling

2.2.1 DNA extraction

DNA (and RNA for transcriptomics – described later) extracted using the Qiagen AllPrep DNA/RNA Mini Kit using specific modifications. Colonic biopsies stored in Tissue All protect tubes at -80C were thawed on ice. Biopsies were then placed into a new sterile Eppendorph and 10 µl of 14.3 M β-mercaptoethanol (β-ME) were added per 1 ml Buffer RLT Plus. A 5mm single TissueLyser bead was added to the eppendorph following which the biopsy was mechanically disrupted and homogenised with the Tissuelyzer at 2 x 3 minutes at 20–30 Hz. Subsequent steps for DNA/RNA extraction were as per the kit protocol.

2.2.2 16S rRNA PCR

DNA was quantified using Qubit and normalised to 10ng/ul. The standardised Earth Microbiome 16S Illumina Amplicon Protocol was used for 16S rRNA PCR (174). Primers 515F–806R targeting the V4 region of the 16S SSU rRNA with single indexing were used. The 16S V4 amplification primers have been modified from the original 515F–806R primer pair that was described by Caporaso et al., 2011 (175). Here Golay barcodes are on the forward primer 515F and degeneracy was added to both the forward and reverse primers to remove known biases against specific microbial taxa. Primers were ordered desalted from Sigma and was resuspended in nuclease free water.

The PCR reaction mixture and thermocycler conditions are outlined as below in Table 2 - 1 and Table 2 - 2. Optimisation was done performed to decide these conditions and are described later in the chapter.

Each sample had two technical replicates (two different barcoded forward primers). Additionally, samples were in amplified in PCR triplicates (i.e.: 3 replicates of 25-µL PCR reactions using the same forward Golay barcoded primer and template DNA). Only PCR triplicates were pooled at the end of the PCR amplification (total volume 75ul). PCR was performed in individual PCR strip tubes for each sample in order to avoid cross contamination. PCR was done in a single batch (for each chapter) and multiple appropriate kit negative controls were used.

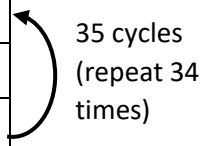
Table 2 - 1 : PCR reaction mixture

Reagent	**Volume**
PCR-grade water	13.0 µL
PCR master mix (2x)	10.0 µL
Forward primer (10 µM)	0.5 µL
Reverse primer (10 µM)	0.5 µL
Template DNA (10ng/ul)	1.0 µL
Total reaction volume	*25.0 µL*

- PCR-grade water from Sigma (cat. no. W3500) or MoBio (cat. no. 17000-11)
- Platinum Hot Start PCR Master Mix (2x) from ThermoFisher (cat. no. 13000014)
- Final master mix concentration in 1x reaction: 0.8x
- Final primer concentration in 1x reaction: 0.2 µM

Table 2 - 2 : Thermocycler conditions

Temperature	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



Post PCR clean up

PCR triplicates for each sample were pooled into a single volume (75 µL) and transferred to a round bottom plate for clean-up. Agencourt AMPure XP beads (Beckman Coulter, California, US) were equilibrated to room temperature. 120 µL of bead solution was added to each library (75 µL) and mixed by pipetting the entire volume at least 10 times. This was then incubated for 5 minutes at room temperature to allow the beads to bind to the 16S amplicons. The plate was then placed on a magnetic rack for 2-4 minutes until the solution appeared clear following which the solution was discarded. With the plate still positioned on the magnetic rack the bead pellet was washed twice with 200 µL of freshly prepared 80 % ethanol. Excess ethanol was discarded by pulse centrifugation of the plate and aspiration along with air-drying the plate for approximately 5-10 min to evaporate the ethanol. The plate was removed from the magnetic rack and 23 µL of nuclease free water was added to each bead resuspended by pipetting. The plate was put back on the magnetic rack for 4 minutes following which 20 µL of the liquid was transferred to a new Eppendorph.

Normalisation

Each library was quantified by Qubit HS DNA (expand). Amplicon fragment lengths of 10% of the samples were measured by Tapestation. Average fragment length of the amplicon was calculated and used for normalisation. The equation for calculating DNA concentration is shown below.

$$\frac{(\text{concentration in ng}/\mu\text{l})}{(660\ \text{g/mol} \times \text{average library size in bp})} \times 10^6 = \text{concentration in nM}$$

Libraries were normalised to 4nM DNA concentration and pooled together. The DNA concentration of the pooled library measured twice, and an average was taken. An example of a 16S library from one of the experiments in the thesis is shown in Figure 2 - 1.

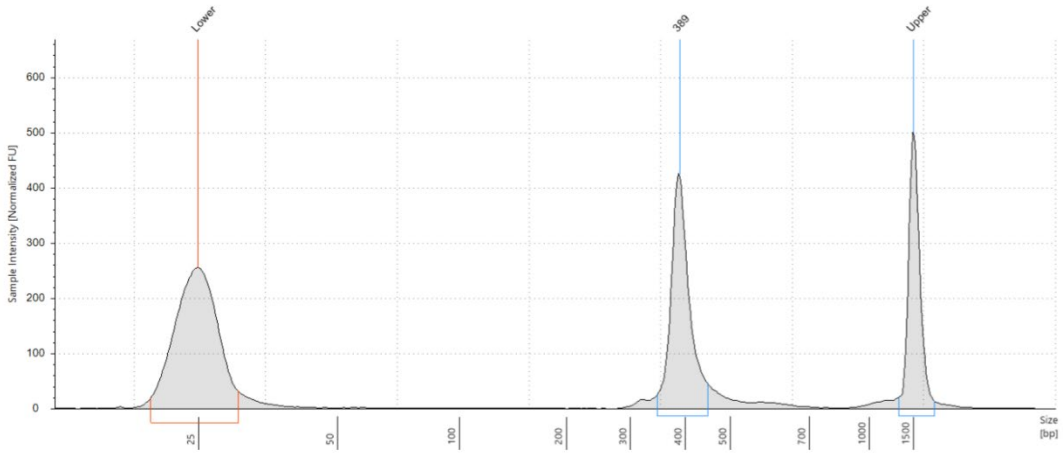


Figure 2 - 1: Representative Tapestation analysis of pooled 16S rRNA library

Average amplicon sizes were at 375 base pairs and library was cleaned up again if there was a presence of primer dimers.

Library denaturation, dilution and sequencing

The standard MiSeq System Denature and Dilute Libraries protocol was followed. Briefly, the pooled 16S library was denaturation with 0.2 N NaOH solution and diluted with appropriate volume of HT1 buffer to achieve a 12 pM library loading concentration. Freshly prepared denatured PhiX control at a concentration of 12.5 pM was prepared and added to the library in appropriate volumes to achieve a 20% spike-in. This high spike in concentration of PhiX was used following previously failed 16S runs with Q30 scores of between 75% to 85% with a lower concentration. This higher spike-in was used to compensate for the low diversity of a 16S rRNA library and consequently gave Q30 scores of greater than 85%.

Paired end sequencing was performed on Miseq Reagent Kit v2 500 cycle kit (250bp x2) on the Illumina MiSeq sequencing platform (Illumina, San Diego, US). Sequencing runs with Q30 scores of less than 85% were repeated.

2.2.3 Bioinformatics analysis for 16S rRNA profiling

A summary of bioinformatics pipeline used is shown in Figure 2 - 2.

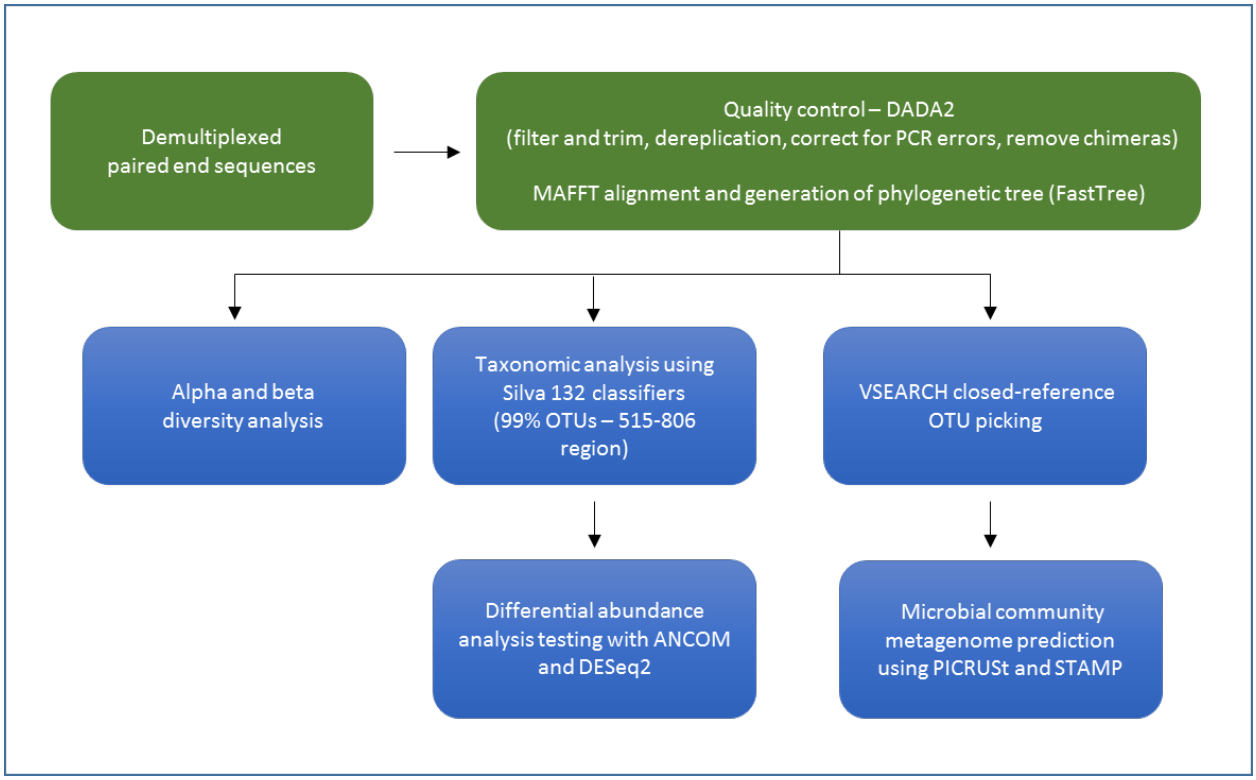


Figure 2 - 2 : Bioinformatics pipeline using the QIIME 2, DeSEq2 and PICRUSt platforms

This workflow outlines the standard QIIME, DeSEq2 and PICRUSt pipeline used for analysis and is detailed in the following sections.

16S rRNA sequence reads filtering and mapping

Demultiplexed FASTQ sequence files were obtained as part of the standard Illumina post sequencing pipeline. 16S rRNA analysis was then performed using the Quantitative Insights

Into Microbial Ecology 2 (QIIME2 - https://qiime2.org/ 2018.3) pipeline (176). Briefly, the FASTQ data were imported and number of sequences obtained per sample and sequence qualities at each position in the sequence data was visualised. Sequence quality control and feature table construction was then performed using the built in DADA2 pipeline. Bases at the start of the reads were trimmed and length of reads were truncated at positions were Q scores were less than 30 for both forward and reverse reads. The DADA2 pipeline then detects and corrects (where possible) Illumina amplicon sequence data and this quality control process will additionally filter any phiX reads that are identified in the sequencing data and will filter chimeric sequences. A tree was then generated from the de-noised amplicon sequence variants (ASVs) for phylogenetic diversity analyses using the built-in mafft program. Here multiple sequence alignment of the sequences in the feature table is performed and filtered to remove highly variable positions (masked alignment). Following this, the pipeline applies FastTree to generate a phylogenetic tree from the masked alignment by inferring approximately-maximum-likelihood phylogenetic trees from alignments.

Analysing microbial diversity

Diversity is a measure that quantifies the number of different states in a system. Diversity is considered an “emergent property” of a community, acting at the level of the community rather than the level of individual species (177). Diversity is also an important measure used in conservation management, as an indicator of “well-being” of ecological systems. There are primarily forms of diversity measurements in understanding distributions of bacterial populations within a community; alpha and beta diversity as illustrated in Figure 2 - 3.

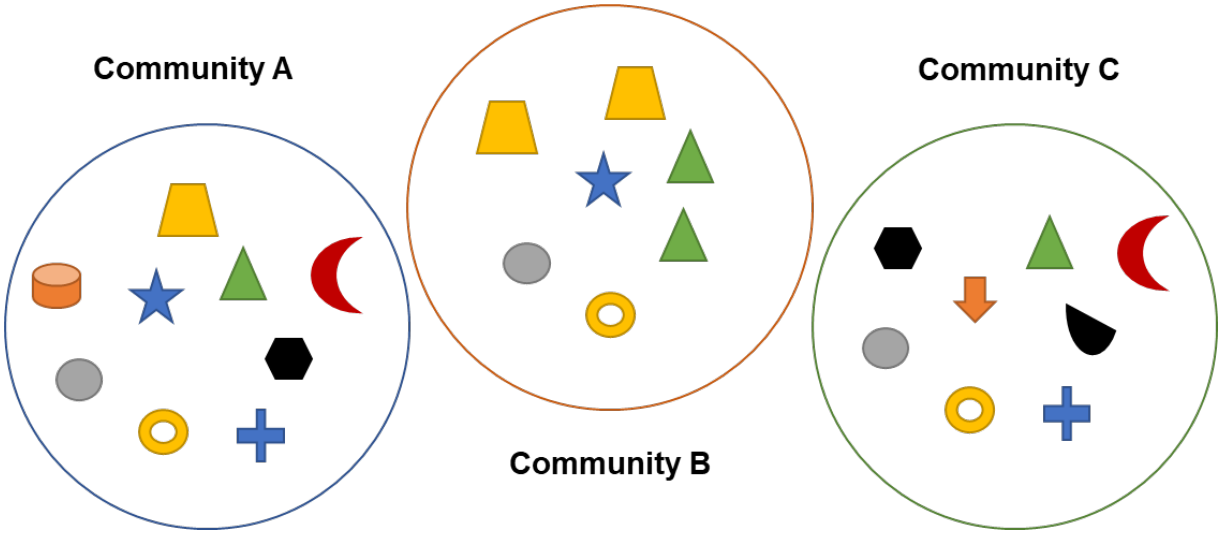


Figure 2 - 3 : Illustrative explanation of alpha and diversity

Alpha diversity of community A is 9 species, community B is 5 species and community C is 8 species. Therefore, based on ecological richness A is more diverse than C which is more diverse than B. Beta diversity is greatest between A and C with 6 species different between them and 5 species in common.

In microbial ecology, alpha-diversity is considered as the diversity within a single sample or set of replicates with a specific cohort being compared (178). Although very simply this can be achieved by counting how many different OTU are in there within a sample, this is typically not possible as it would require identification of every single unique taxon in a microbial sample (richness) whilst taking into account the incompleteness of the community (evenness). One way to estimate the true richness of a sample is to take into account the tail of the species (or OTU) abundance distribution, more specifically the number of singletons (species observed once) and doubletons (species observed twice). This is done by several alpha diversity measurement indices, however Shannon diversity and Chao1 are commonly used. Shannon diversity index combines richness and diversity by measuring both the number of species and the inequality in abundances between species. A large value would therefore be given due to the presence of many species with equally balanced abundances.

Values can range from one (in case of a single dominant species) to the total number of all species (in case of all species having equal abundance). Chao1 index however estimates diversity from abundant data and takes into account the number of rare taxa missed from under sampling.

Beta diversity measures variation of microbial communities between samples (179). Beta diversity focuses on taxonomic abundance profiles to observe the differences between microbial communities between different environments. The choice of beta-diversity metric can potentially have important consequences to subsequent analyses, such as clustering and ordination (statistical techniques by which data from a large number of populations or cohorts are represented as points in a multidimensional space). This is due to the interaction between distance metrics and techniques for normalisation of data, which can widen or reduce the apparent distance between samples. Different approaches to community dissimilarity, such as OTU-based vs. phylogenetic, may highlight different aspects of the community and its functioning.

16s rRNA diversity analysis

Samples were rarefied based on minimum sequencing depth of 25,000 and diversity analysis was performed with the q2-diversity plugin. Alpha diversity metrics were assessed for community-based species richness and evenness by measuring Chao1 (estimates diversity from abundant data and number of rare taxa missed from under sampling), Shannon index (calculates richness and diversity using natural log thereby accounting for both abundance and evenness of the taxa present) and Faith's Phylogenetic Diversity (measures biodiversity that incorporates phylogenetic difference between species). Paired and unpaired beta-diversity was assessed by calculating weighted and unweighted UniFrac

(weighted accounts for abundance of observed organisms) and Bray-Curtis dissimilarity for qualitative and quantitative assessment of microbial community dissimilarity and phylogenetic relationships. Three-dimensional principal coordinate analysis (PCoA) was generated using EMPeror software and Monte-Carlo simulations (1000 permutations) were done to calculate a p-value.

Differential taxonomic abundance analysis

Taxonomy was assigned to ASVs against the Silva 132 99% OTUs (515-806 region) database with a supervised learning classifier. Statistically significant differences in the relative abundance of taxa associated with patient cohorts were performed using linear discriminant analysis (LDA) effect size (LEfSe) via the Galaxy platform (180). Only taxa with LDA greater than 2 at a p value <0.05 were considered as significantly enriched.

Predictive metagenomics

The functional profiles of microbial communities were inferred by using PICRUSt2 -derived relative MetaCyc and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway abundance information and analysed through STAMP (181-183).

Optimisation of 16S rRNA PCR for DNA obtained from colon biopsies

Both optimum input template DNA concentration and number of PCR cycles for DNA extracted from colon biopsies were decided through prior experimentation. Here input DNA concentrations of 1ng/ul, 2ung/ul, 5ng/ul and 10ng/ul were put through 33, 35, 37 PCR cycles. The rest of the 16S rRNA PCR protocol was unchanged. The amplicons obtained were then run through an agarose gel for DNA electrophoresis and assessed against a

positive control (DNA (1ng/ul) from *Salmonella typhimurium*). The highest intensity and sharpest band with the least noise was seen at 10ng/ul at 35 cycles and consequently this was used for 16S rRNA PCR.

2.3 Colonic mucosal and peripheral blood immunophenotyping

2.3.1 Lamina propria cell isolation from colon biopsies

Three pairs of colon biopsies were collected in gentleMACS C tubes containing complete RPMI on ice. A collagenase-DNase digestion mix with 100 units/mL of collagenase and 150 µg/mL DNase was added to the gentleMACS tubes and incubated at 37°C for one hour. Cells in the biopsies were then dissociated further by gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) on the 'E program' for 1 minute. The cell suspension was then passed through a 70um cell strainer and washed with PBS.

Cells were then centrifuged for 8 minutes at 1400 RPM with full brake at room temperature and supernatant discarded. Percoll at dilutions of 40% solution and an 80% solution were prepared by diluting 100% Percoll solution with appropriate volumes of complete RPMI. The pellet was re-suspended in 5 mL of the 40% Percoll solution and pipetted to mix following which the cell suspension was transferred to a new 15 mL conical tube. Using a Pasteur pipette 5mL of the 80% Percoll solution was carefully underlaid below the 40% Percoll solution. The tube was then spun for 20 minutes at 1400 RPM at room temperature with the brake off. Using a 3 mL transfer pipette, the interphase was extracted and transferred to a new 15 mL conical tube containing with 10mL 1X PBS. The cell suspension was then centrifuged for 8 minutes at 1400 RPM at 4°C. The supernatant was aspirated carefully

down to the pellet level following which pellet was resuspended in the 200 uL of complete RPMI and placed on ice. Viable cells were counted using Trypan Blue stain with a dilution factor of 4 and a haemocytometer. The protocol was optimised to obtain at least 1.5 million cells from 3 pairs of colon biopsies. Although the cells obtained may carry over intraepithelial lymphocytes (IEL), the density gradient centrifugation and subsequent gating strategies would have removed a significant contaminating IEL fraction as demonstrated in published protocols (184).

2.3.2 PBMC isolation from whole blood

PBMCs were isolated from whole blood by standard density centrifugation. 6mls of blood was collected from patients in EDTA tubes containing heparin. The whole blood was then transferred to a 50mls Falcon tube and diluted in 1 x PBS in a ratio of 1:1. In two 15mls Falcon tubes, 6mls of Ficoll Histopaque was then added and then 6mls of diluted blood was then carefully layered on top (giving a ratio of 1:1). The tubes were then centrifuged at room temperature for 20 minutes at 800 RPM with the brake set to 1. PBMCs were then harvested from the interphase layers (between plasma and Ficoll Histopaque), washed twice with PBS by centrifugation for 8 minutes at 1400 RPM with full brake. Cells were resuspended in complete RPMI and counted using a haemocytometer as described above.

2.3.3 Stimulation of cells for cytokine analysis

For intracellular cytokine staining panels cells were stimulated with PMA / inomycin followed by blocking by a protein transport inhibitor Brefeldin A. For these 500,000 cells from the cell suspension were transferred into a new 15ml Falcon tube and volumes adjusted (by

centrifugation and resuspension) to were resuspended in 500 ul of complete RPMI (to achieve a desired concentration of 1 x 10^6 cells/ml). 1ul of Cell Activation Cocktail (Biolegend) consisting phorbol-12-myristate 13-acetate (40.5 μM), ionomycin (669.3 μM) and Brefeldin A (2.5 mg/ml) was added to the cell suspension and mixed by gentle vortexing. 100ul (containing approximately 100,000 cells) were transferred to 5 wells in a 96 well round bottom plate. The plate was then incubated at 37°C in a CO2 incubator for 4 hours. The activated cells were then harvested by centrifugation at 1500 RPM for 5 minutes and washed once with 1 x PBS and resuspended in 200 ul of 1x PBS. Cells were then transferred to a new round bottom plate for staining.

2.3.4 Extracellular, intracellular and intranuclear marker staining

Cells were transferred to round bottom plates and with the number of wells were determined by the panels (see below). Approximately 500,000 cells/well were used for subset panels and 100,000 cells/well for control fluorescent minus one panel. Cells were centrifuged at 1500 RPM for 3 minutes at 4oC and supernatant discarded by flicking the plate upside down over a sink.

Live dead antibody staining: 100 ul of Live/Dead antibody mix (diluted at 1:1000 in 1 X PBS) was added to each well following which the plate was covered with foil and incubated on ice for 20 minutes. A further 100ul of 1 x PBS was added at the end of the staining period for washing (centrifuged at 1500 RPM for 3 minutes at 4oC) and the wash was repeated again with 100ul of PBS. Cell surface marker staining (panels defined in the next section): 100ul of pre-prepared cell surface cocktail (in staining buffer) was added to the appropriate wells and pipetted to mix. The plate was covered with foil and incubated on ice for 30 minutes following which the cells were washed twice with staining buffer.

Fixation and permeabilization: The BD FoxP3 kit was used for fixation and permeabilization. Following the final wash, the supernatant was discarded and pulse vortexed to dissociate the pellet. 200 µL of Foxp3 Fixation/Permeabilization working solution (1 part of Foxp3 Fixation/Permeabilization Concentrate with 3 parts of Foxp3 Fixation/Permeabilization Diluent) was added to each well and pipetted to resuspend the pellet. The plate was covered with foil and incubated on ice for 30 minutes after which the plate was centrifuged at 1500 RPM for 3 minutes and supernatant discarded. 200 µL 1X Permeabilization Buffer (1 part of 10X Permeabilization Buffer with 9 parts of distilled water) to each well and the plate was centrifuged again at 1500 RPM for 3 minutes. The wash was repeated, and cells were then resuspended in 100 ul of Permeabilization Buffer containing relevant intracellular or intranuclear fluorochrome conjugated antibodies at appropriate dilutions. The plate was covered with foil and incubated overnight in a fridge (4oC). A further 100ul of Permeabilization Buffer was then added to each well and centrifuged. The pellet was then re-suspended in 150 ul of PBS and kept at 4C for up to 2 days until acquisition by flow cytometry.

Compensation staining controls

UltraComp eBeads™ Compensation Beads were used for compensation staining controls and sufficient beads were added to ensure at least 10,000 events were recorded during flow cytometry.

2.3.5 Flow cytometry

Stained cells were then acquired on BD Fortessa Flow Cytometer (BD, New Jersey, US). Flow analysis was performed using FlowJo software version (Tree Star, Oregon, US). Prism 8 software (Graphpad, California, US) was used for statistical analysis and graphical representation. Statistical significance was determined by t tests and multivariate analysis performed by regression analysis.

2.3.6 Immunophenotyping panels and representative gating strategy

Antibody panels were designed and optimised for T cell immunophenotyping specifically focusing on CD4 T cells. In order to maximise quality of gating on CD4 subsets in view of both the limited number of LPMC cells obtained from colon biopsies along with threshold of fluorochrome antibody panels within a single flow cytometry assay, I did not explore additional CD8 subtypes or ILC subsets.

- Panel 1 - CD4 subset panel: Th1 defined as CD4+CD161-CCR6-CXCR3+CCR5+, Th2 defined as CD4+CD161-CCR6-CXCR3-CCR5-, Th17 defined as CD4+CD161+CCR6+ and Treg defined as CD4+CD25+CD127-FoxP3+ and CD8 Tregs defined as CD8+CD25+CD127-FoxP3+. FMO for CD161 and CCR6 were used to identify gates. Representative gating shown in Figure 2 - 4.

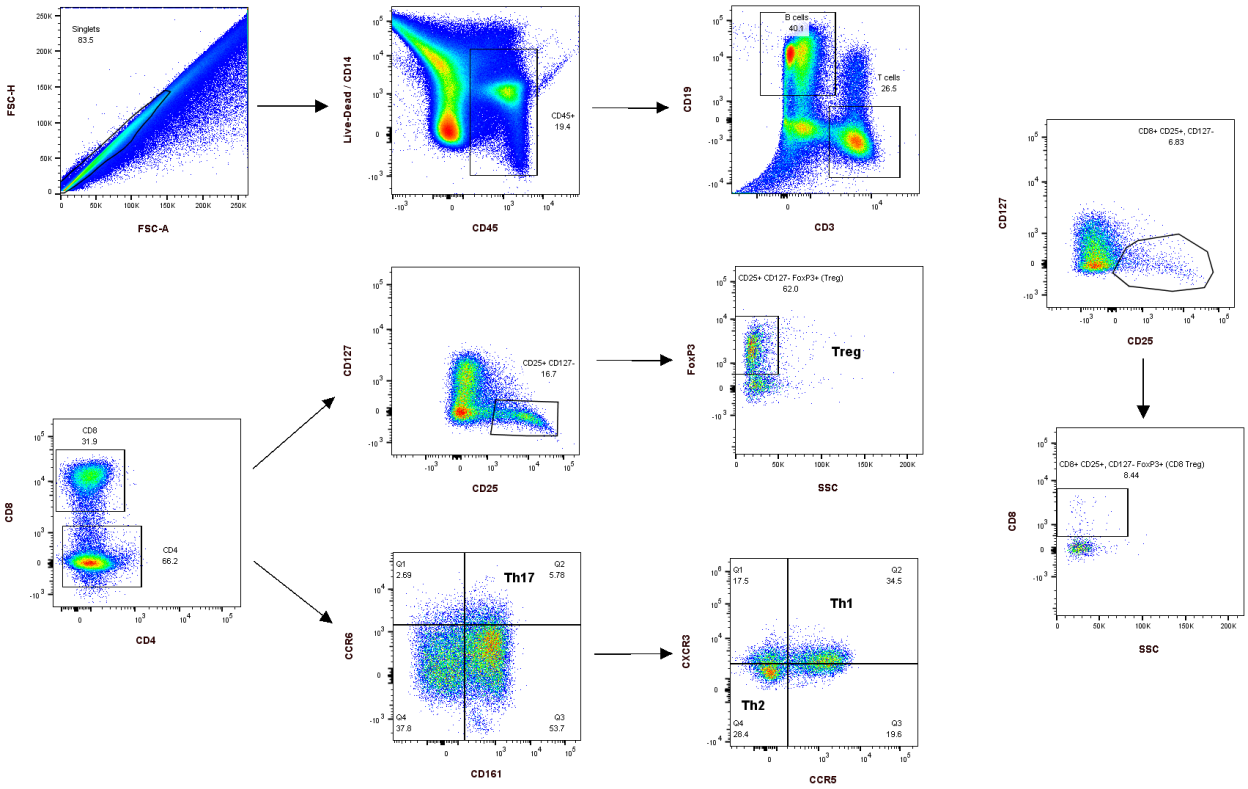


Figure 2 - 4 : Representative gating strategy for CD4 subsets panel.

Subset definition as above. FMO for CD161 and CCR6 were used to identify gates.

- Panel 2 - CD4 cell intracellular cytokine panel: This panel consisted of Th1 cytokines: TNFα, IFNγ, Th2: 1L3, IL5, Th17: IL-17 and Treg: IL-10. Cytokines were gated on CD4 cells. Cytokine FMOs were used to identify where gates were drawn. The full panel was used for Chapter 5, however only TNFα, IFNγ and IL-17 were used for Chapter 4 and 6. Representative gating shown in Figure 2 - 5.

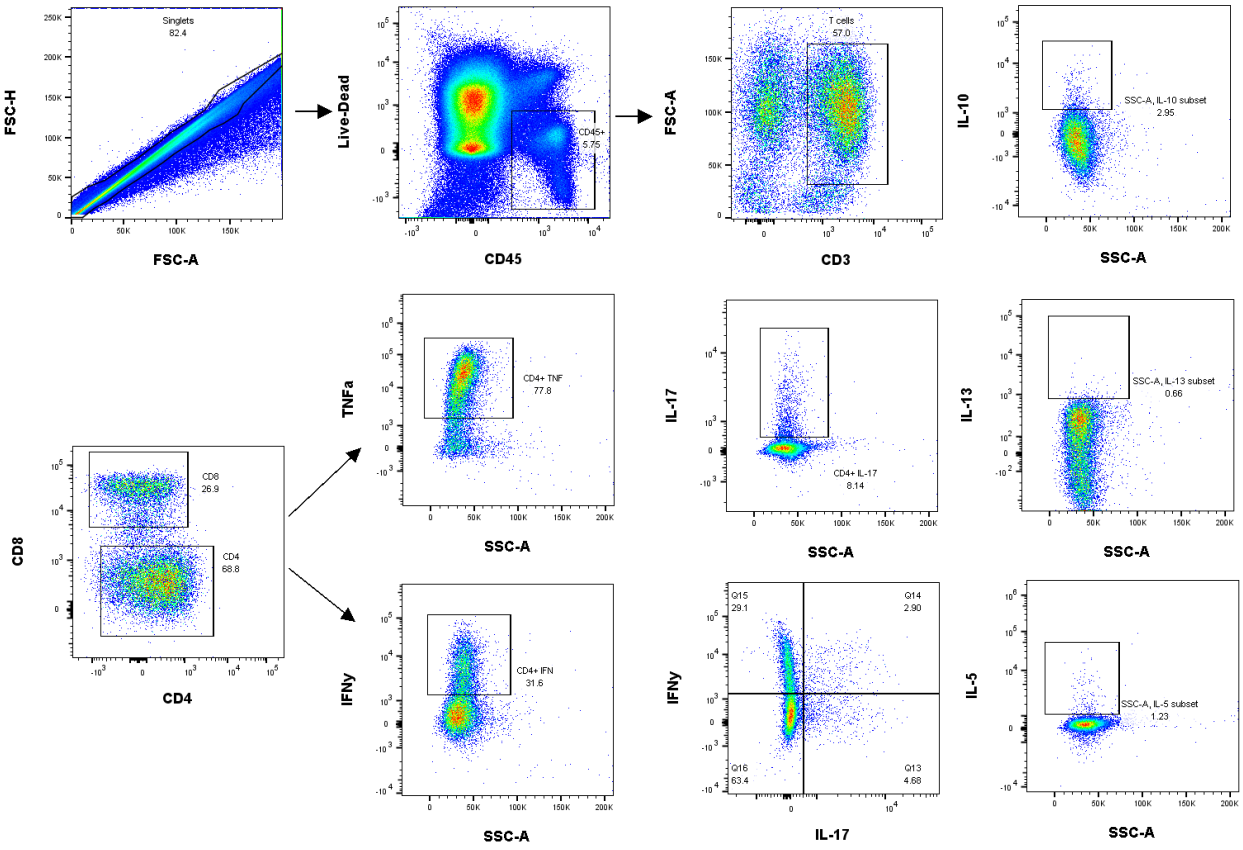


Figure 2 - 5 : Representative gating strategy for cytokine producing CD4 panels.

Subset definition as above. Cytokine FMOs were used to identify where gates were drawn.

- Panel 3 (Chapter 5 only) - Treg panel: Tregs defined as CD14-CD19- CD45+CD3+CD8-CD4+ CD25+CD127-, naïve Tregs defined as CD14-CD19- CD45+CD3+CD8-CD4+ CD25+CD127-CCR7+CD45RA+, central memory Tregs defined as CD14-CD19-CD45+CD3+CD8-CD4+ CD25+CD127-CCR7+CD45RA-, effector memory Tregs defined as CD14-CD19- CD45+CD3+CD8-CD4+ CD25+CD127-CCR7-CD45RA- and gut homing Tregs defined as CD14-CD19- CD45+CD3+CD8-CD4+CD25+CD127-CCR7-CD45RA-alpha4+. FMO for CCR7, CD45RA and alpha4 were used to define gates. Representative gating shown in Figure 2 - 6. Additionally, Ki67, CTLA4. BCL2 and CD39 antibodies were used (gated on Tregs) for the last 7 patients in Chapter 5.

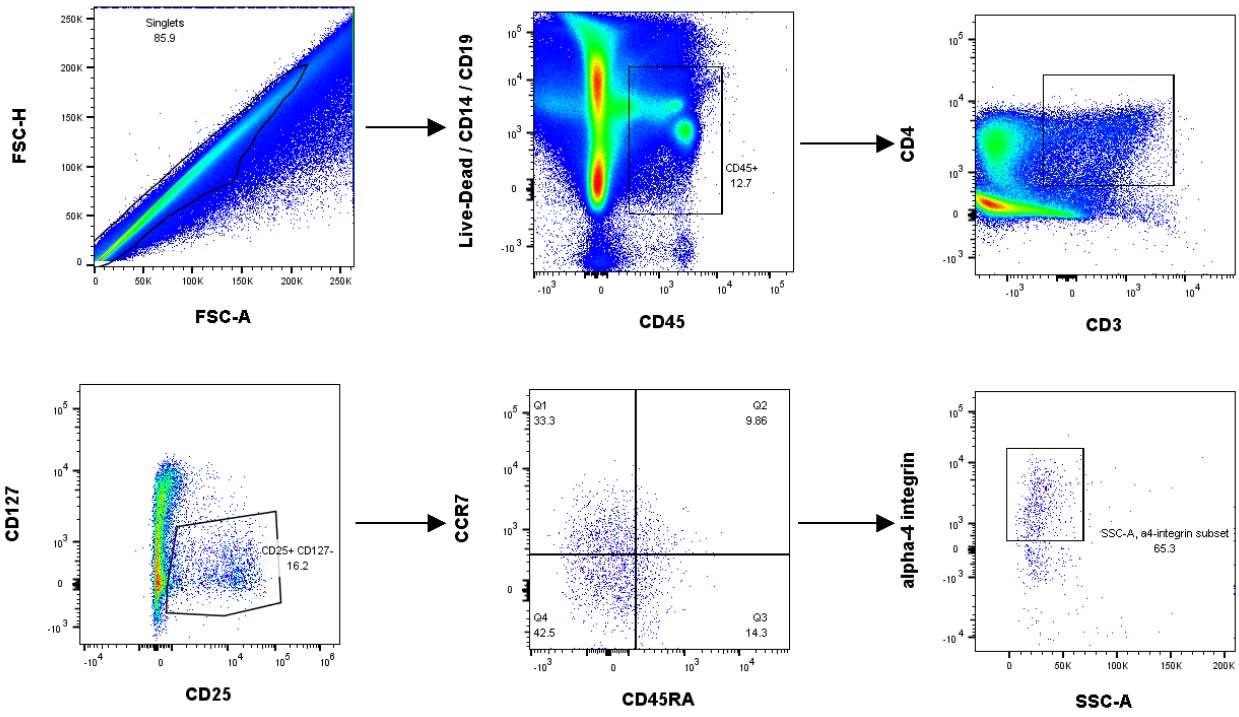


Figure 2 - 6 : Representative gating strategy for regulatory T cell subset panel.

Subset definition as above. FMO was used to define CCR7, CD45RA and alpha-4 integrin gates.

Antibody optimisation

Concentrations of antibodies in this study were either optimised before being used in the assays or based on previously established optimum concentrations for colonic LPMCs. An example of antibody optimisation undertaken is shown in Figure 2 - 7.

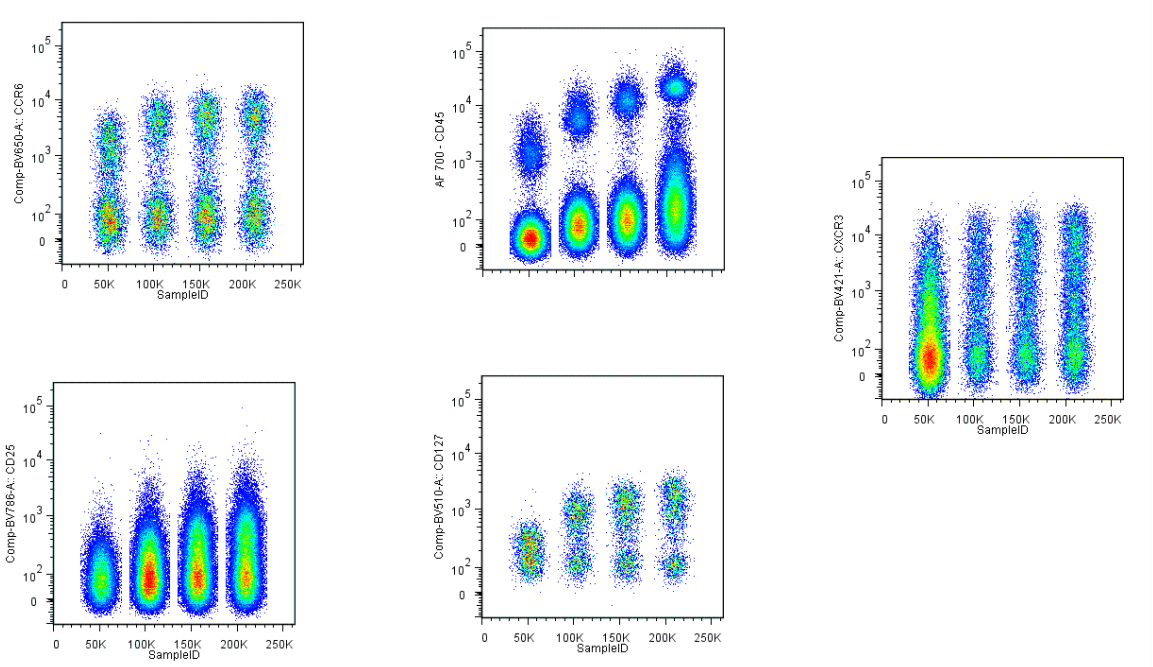


Figure 2 - 7 : Example of antibody optimisation

Antibodies were optimised prior to use or based on previously established optimum concentrations for colonic LPMCs.

Antibodies used in the study

The antibodies used in the study are detailed below in Table 2 - 3

Table 2 - 3 : List of antibodies used

Antibody	Fluorochrome	Supplier	Catalogue Number
Live-Dead	APC-CY7	eBioscience	65-0865-14
Bcl2	AF647	Biolegend	658705
CCR5	PE-Texas	Biolegend	359126
CCR6	BV650	Biolegend	353425
CCR7	PeCF594 (PE-TR)	BD Biosciences	562381
CD127	BV510	Biolegend	563036
CD127	PE-CY7	Biolegend	351319
CD14	APC-CY7	Biolegend	325620
CD161	BV711	BD Biosciences	563865
CD19	PE	Biolegend	302208
CD19	APC-CY7	Biolegend	302217
CD25	BV785	Biolegend	302638
CD25	BV421	BD Biosciences	562443
CD3	FITC	Biolegend	344804
CD3	BV605	Biolegend	317322
CD3	BV605	Biolegend	317322
CD39	BV711	BD Biosciences	563680
CD4	PerCP	Biolegend	300528
CD4	BV650	BD Biosciences	563875
CD4	APC-CY7	Biolegend	300517
CD45	AF700	Biolegend	304023
CD45	BV510	BD Biosciences	563204
CD45	AF700	Biolegend	304023

Table continued…

Antibody	Fluorochrome	Supplier	Catalogue Number
CD45RA	AF700	Biolegend	304119
CD45RA	BV785	Biolegend	304139
CD49d (a4 integrin)	PE	eBioscience	12-0499-42
CD8	PE-CY7	Biolegend	344711
CD8	PECF594	BD Biosciences	562282
CTLA4	BV786	BD Biosciences	563931
CXCR3	BV421	Biolegend	353715
FoxP3	APC	eBioscience	17-4776-41
IFNy	BV650	Biolegend	502537
IL-10	PE	Biolegend	501414
IL13	BV711	BD Biosciences	564288
IL-17	FITC	Biolegend	512303
IL-5	BV421	Biolegend	504311
Ki67	PerCP	Biolegend	652423
Live-Dead	BV510	BD Biosciences	564406
TNFa	APC	Biolegend	502912

2.4 Colonic mucosal transcriptomics

Whole RNA sequencing was performed for experiments in Chapter 6 and 3' RNA sequencing was performed for experiments in Chapter 5.

2.4.1 RNA extraction from colon biopsies

Genomic DNA and RNA were extracted from mucosal biopsies within 2 weeks of collection as per the protocol described in section 2.2.1. Briefly, Qiagen RNAlater TissueProtect tubes containing mucosal biopsies were thawed on ice. Following mechanical lysis with TissueLyser (Qiagen, Hilden, Germany), the Qiagen AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) was used for extraction of DNA and RNA from biopsies. On column DNase digestion was done using RNase-Free DNase Set (Qiagen, Hilden, Germany) prior to elution of RNA in order to reduce carry-over of contaminating DNA.

2.4.2 Quality control checks

The RNA was quantified by Qubit (Thermofisher, Massachusetts, US) and quality checked by Tapestation (Agilent, Santa Clara, US). Samples with an RNA integrity number (RIN) of less than 8 were excluded. An example of a RIN obtained following optimisation of RNA extraction from colon biopsies is shown in Figure 2 - 9.

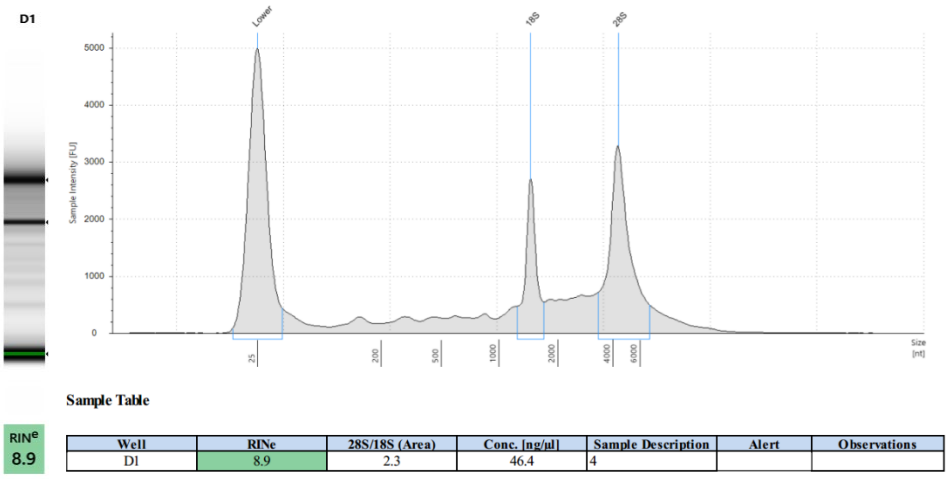


Figure 2 - 8 : Representative Tapestation analysis of RNA integrity

Samples with an RNA integrity number (RIN) of less than 8 were excluded

2.4.3 Optimisation of ribo-depletion

As part of RNA-seq protocol optimisation, MICROB*Enrich* (Invitrogen), MICROB*Express* Kits (Invitrogen) and Ribo-Zero Gold Epidemiology rRNA Removal Kit (Illumina, San Diego, US) were used initially for ribosomal RNA depletion (ribo-depletion) in multiple round of experiments. Analysis of 18S and 26S ribosomal RNA peaks on the Tapestation demonstrated that ribo-depletion was most efficient with the Ribo-Zero Gold Epidemiology rRNA Removal Kit with nearly 95% removal of ribosomal RNA (Figure 2 - 8). This was further confirmed in sequencing runs that followed that showed only 3% to 4% alignment of raw sequence reads to the ribosomal RNA database. A summary of the ribo-depletion experiments with different kits is shown in Figure 2 - 9. Samples were then cleaned up by ethanol / glycogen precipitation using Pellet Paint® Co-Precipitant (Merck Millipore). Fragment sizes and RIN was checked again after ribodepletion to ensure there was significant reduction in 18S and 23S peaks. If there were significant ribosomal peaks, the ribo-depletion protocol was repeated.

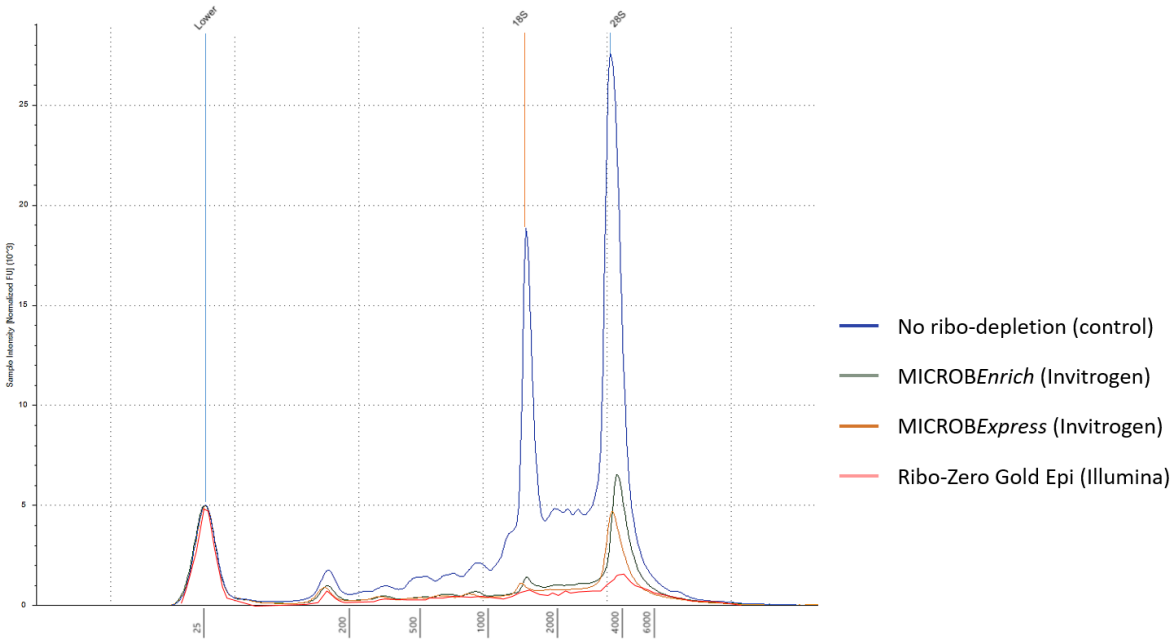


Figure 2 - 9 : Tapestation graphs showing differences in ribodepletion of RNA extracted based on kits used.

Ribo-Zero Gold Epidemiology kit showed the greatest reduction in ribosomal RNA peaks.

2.4.4 RNA-seq library prep

The SMARTer Stranded RNA-Seq Kit (Takara, Kusatsu, Japan) was used for cDNA synthesis and strand-specific library construction. This uses SMART (Switching Mechanism at 5' End of RNA Template) chemistry to allows the efficient incorporation of known sequences at both ends of cDNA during first-strand synthesis, without adapter ligation. 90ng of input RNA was used and standard protocol for RIN > 8 was followed as per kit instructions. Clean-up was performed using AMPure XP beads at a 1.8:1 bead to DNA library ratio. The final libraries were eluted in 20ul of elution buffer and fragment sizes assessed again using the Tapestation to ensure there were no primer dimers. For samples with primer dimers (as shown in Figure 2 - 10) a further clean-up was performed with AMPure XP beads at a 2:1 bead to DNA library ratio. Libraries were pooled and 75 bp paired-end sequencing was performed using NextSeq 500/550 High Output Kit v2 kits

(Illumina, San Diego, US). Only 10 libraries were sequenced at a time on a single run in order to obtain a minimum of at least 45 million PE reads for each sample.

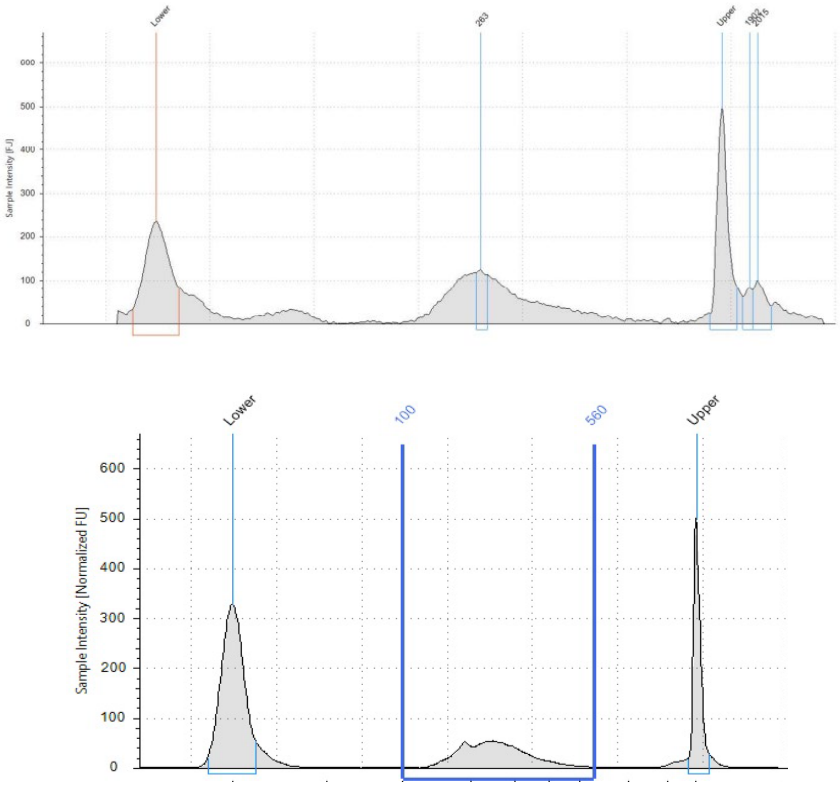


Figure 2 - 10 : Example of removal of primer dimers in RNA-seq libraries.

The Tapestation QC shows that disappearance of the primer dimer peak after clean up.

2.4.5 3’ RNA-Seq

3’ RNA-Seq was performed on colon biopsies obtained from STOP-Colitis trial (Chapter 5). Briefly RNA was extracted using the standard protocol described in 2.4.1. RNA was quantified and QC performed on Tapestation to ensure RIN of at least 8. The QuantSeq 3' mRNA-Seq Library Prep Kits (Lexogen, Vienna) was used in an automated work flow

(Hamilton Robotics) for 3' RNA-Seq library prep. The normalised libraries were then sequenced using the NextSeq 75bp (single end read) high output kit.

2.4.6 RNA sequencing bioinformatics pipeline

The pipeline used for RNA sequencing is summarised below in Figure 2 - 11 and detailed in the subsequent sections.

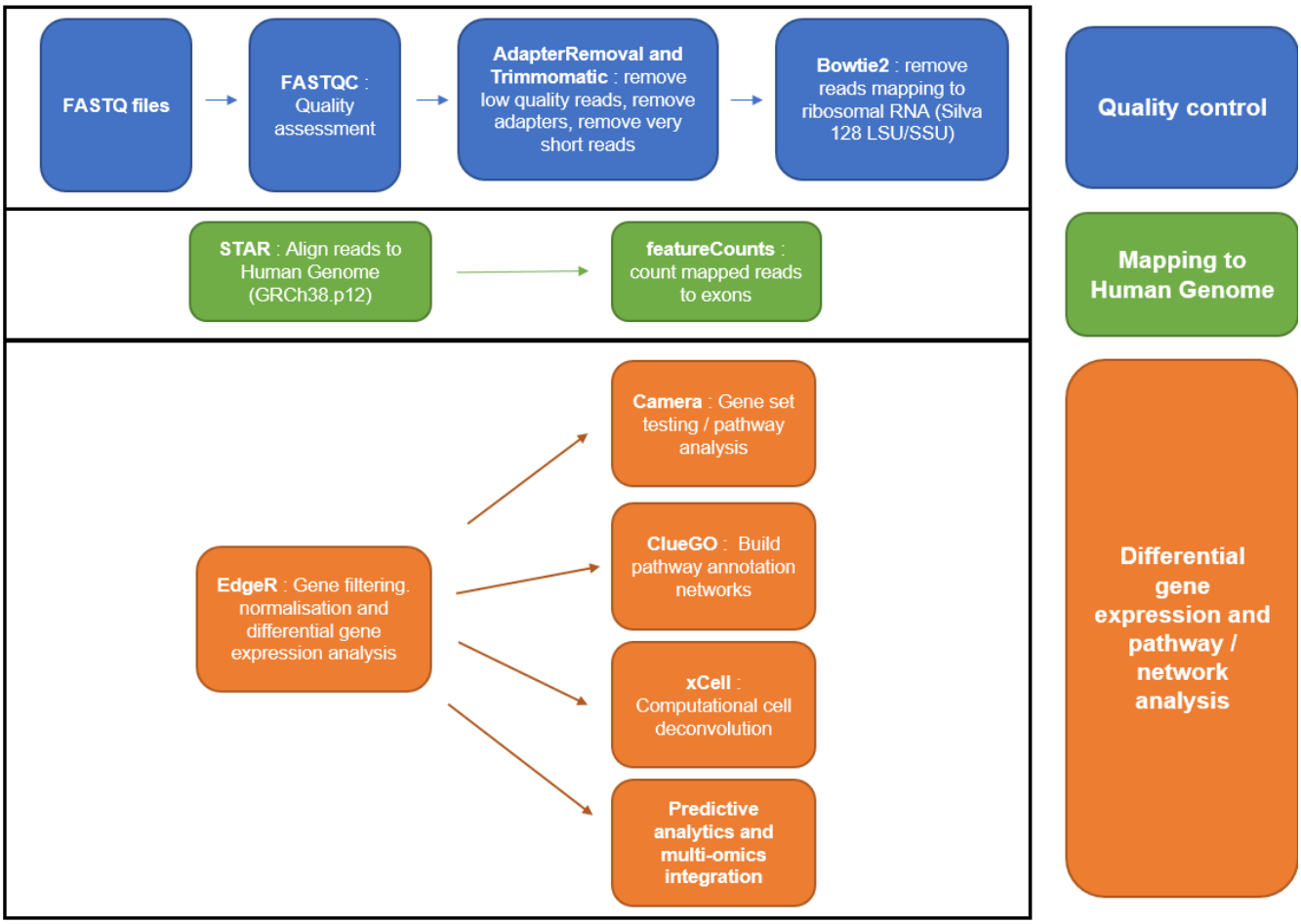


Figure 2 - 11 : Pipeline used for RNA sequencing

Flow chart demonstrating different bioinformatics steps for quality control, human genome mapping and differential gene expression and pathway analysis.

RNA sequence reads filtering and mapping

Reads obtained were first quality checked with FastQC in order to obtain a quick impression of whether the raw sequence data has any major problems before proceeding with the analysis (1). Following this AdapterRemoval v2 was used to search and remove Illumina adapter sequences, sequences with 'N's' and to trim low quality bases from the 3' end of the reads (185). A further quality control step was performed with Trimmomatic v0.39 to allow a 'Sliding Window' trimming (4:20) based on quality of reads quality within a window falling below a threshold along with cutting out the Smarter Stranded Oligo that were attached during the library prep as part of the protocol (186). Contaminating ribosomal RNA reads were removed by mapping to the Silva SSU/LSU ribosomal RNA database (bacteria, archaea, and eukarya) using Bowtie2 using the '—very-sensitive alignment option' (187, 188).

Paired end reads that were not aligned to ribosomal RNA database were outputted and subsequently mapped to the human genome database (GRCh38 - patch release 12) using the splice aware aligner STAR (v2.5.3) (189). Highly restrictive custom parameters to reduce false alignments, short read alignments and multi-mapping as defined below:

- --outFilterMismatchNmax 1 (allows only one nucleotide mismatch per read)
- --outFilterMismatchNoverLmax 0.04 (Only one mismatch allowed if read length is less than 50bp)
- --outFilterScoreMinOverLread 0.9 --outFilterMatchNminOverLread 0.9 (alignments shorter than query gene x0.9 length are discarded)

Aligned reads were output as BAM files and quantified with a read summarization program featureCounts (190). In order to address multimapping of reads and gene overlaps the parameters '- M - O –fraction' was used to count them as fractions based on query overlapping contribution rather than exclude them. Additionally, to avoid short read

alignments the parameter '-d 25' was used to only count reads that had a minimum acceptable read alignment of 25bp.

2.4.7 Differential gene expression analysis

Gene expression across all samples was evaluated with the Bioconductor package edgeR in Rstudio (25, 191). Genes that were weakly / lowly expressed were filtered out (less than 10 counts per million in at least two libraries) as they are highly unlikely to provide any biological evidence for differential expression. Varying sequencing depths as represented by differing library sizes were addressed by normalisation for RNA composition using the 'calcNormFactors' function. Multidimensional scaling plots were graphed 'plotMDS.DGEList' function. Generalized linear models analysis was used to estimate dispersion in view of the non-normally distributed response data. Once dispersion estimates were obtained and negative binomial generalised linear models were fitted, testing procedures for determining differential expression was performed using the quasi-likelihood (QL) F-test. Differentially expressed genes were only considered significant based on an FDR correction of p value of ≤ 0.05.

2.4.8 Pathway analysis

Gene ontology (GO) and KEGG / Reactome pathway analysis was conducted using Camera for competitive gene set testing (192-195). This molecular pathway analysis tool was used as it consider inter-gene correlation for gene set testing. Differentially expressed pathways were only considered significant based on an FDR correction of p value of ≤ 0.05. Gene ontology hierarchically organised set of standardised terms for biological processes, molecular functions and cellular components, as well as curated and predicted gene annotations (196). Reactome, on the other hand, provides molecular details of signal

transduction, transport, metabolism and other cellular processes as network of molecular transformations in a single data model as a metabolic map (194).

ClueGo was used to build functionally grouped gene ontology and pathway annotation networks in Cytoscape (197). This enables biological interpretation of large lists of genes by integrating GO terms as well as KEGG/BioCarta pathways to create a functionally organised GO/pathway term network.

2.4.9 Computational cell deconvolution

To estimate proportions of cell subsets, we utilized xCell for cell deconvolution (198). This computational method is able to infer multiple cell types based on their gene signatures by reducing associations between closely related cell types. This was used to identify epithelial cells, $CD4^+$ T cells, $CD8^+$ T cells, B cells, dendritic cells and monocytes. Mann-Whitney U tests were used to identify significantly different cell types between the three cohorts.

2.5 Predictive analytics and multi-omic integration

Predictive analytics and multi-omic integration was performed by Dr Animesh Acharjee at the Institute of Computational Biology, University of Birmingham. A summary of the methodology used in described below.

2.5.1 Predictive analytics

We used the Random Forest (RF) machine learning ensemble method to obtain predictive performance of all the features from the transcriptomics, immunophenotype and 16S rRNA microbial profiling datasets (199). This allowed all the features to be analysed in a nonlinear way rather than solely in a linear way, and hence allowed the discovery of more complex dependencies among features. Random Forest uses a bootstrapping method for creating a model (called as training set) or for testing the performance of the model. The bootstrapping process generates random samples from the dataset with replacement. Every bootstrapped sample has a corresponding left out or 'out-of-bag' (OOB) sample which is used to test performance of the algorithm. For example, if we generate 1000 bootstrapped samples, each time we will get a set of predictions from the training samples. The final prediction is simply the average of all 1000 predictions from the trees that do not contain training samples in their respective bootstrap sample (test samples). We used RF as a classification method to classify different response, class labels or outcome variables. The class labels or outcome variables were considered as a combinatorial way: PSC-IBD vs. HC; UC vs. HC and PSC-IBD vs. UC patients. For the classification model, RF needed to use some of the parameters to be set a priori. For example, the number of trees (ntree) and the number of variables (for example: number of genes) randomly sampled as candidates at each split (mtry) needed to be defined. We used ntree=500 and mtry =square root of variables in our models. For example, for this data set, mtry value was set to the nearest integer to the square root of the number of features. To select the optimum number of features from each of the data sets,

we ranked all the features (each data separately) and gradually took top features and estimated AUC values. We took top features that provided the highest AUC value. This process yielded probable predictive features for further analysis and data integration.

2.5.2 Network analysis

We explored selected genes from the RF analysis by mapping to functional information using from three databases: IntAct, KEGG and TRRUST. Based on the gene interactions we took a cut off of at least 15 connections/interactions along with genes that we biologically relevant following which they were integrated with significant immunological parameters and 16s microbial profiles. Data integration was done based on Pearson correlation analysis. Network analysis was done in R (v3.4.3) using qgraph package (200).

CHAPTER 3

Association of gut microbiota with inflammation in ulcerative colitis

3.1 Abstract

Background: Disturbance in gut microbiota (dysbiosis) is a characteristic feature of ulcerative colitis (UC). It is unclear whether dysbiosis contributes to disease by driving immune dysregulation and mucosal inflammation and whether this is a “field effect” in the colon or varies from one site to another.

Method: Paired biopsies from areas of inflamed and non-inflamed colon from 15 patients with active UC were collected. Gut microbiota was characterised using 16S rRNA gene sequencing. We used hierarchical clustering and random forest predictive modelling method to rank the OTUs that distinguish inflamed and non-inflamed mucosa.

Results: Alpha and beta diversity indices were similar at inflamed and non-inflamed sites. Analysis of microbiota at inflamed segments of the colon indicated that *Clostridiaceae* were significantly less abundant (log2FC = 5.6, p = 0.049) and *Atopobiaceae* more abundant at family level analysis (log2FC=7.78, p = 0.005). There was a trend towards a reduction in short chain fatty acid producers and increase in pathogenic bacteria at sites of inflammation. Bacterial functions as predicted based on 16S rRNA gene profile indicated that genes associated with bacterial chemotaxis, motility and flagellar assembly were enriched in the inflamed tissue ($p < 0.02$). These observations were present regardless of Mayo endoscopic subscore, disease extent or use of immunomodulators.

Conclusions: *Clostridiaceae* were significantly underrepresented at sites of mucosal inflammation highlighting the role of butyrate producers in regulation of inflammation. Genes associated with mucosa invasion and immune activation are potentially enriched at sites of inflammation.

3.2 Introduction

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, affects over 750,000 people in the UK and has an increasing incidence worldwide (1). IBD is characterised by chronic intestinal inflammation as a consequence of a dysregulated immune response in a genetically and environmentally predisposed individuals (201). Studies have consistently reported disturbed gut microbiota (dysbiosis) in IBD, however definitive cause and effect relationships have been challenging to prove outside of controlled tissue culture and mice model experiments (8, 23, 202, 203).

To date, the strongest evidence for the role of gut microbiota in contributing to the pathogenesis of IBD in humans comes from faecal microbiota transplantation studies, with meta-analysis demonstrating a clinical response of up to 40% (125, 204). These studies have not explored causative relationships between alterations in microbial communities and modulation of inflammation. Studies exploring transcriptomic analysis of patients with IBD have demonstrated that pathways associated with immune and inflammatory response, cell adhesion and chemotaxis were shown to be significantly enriched at sites of inflammation (181, 205). It is unclear if these processes contribute to, or are a result of this observed dysbiosis at sites of inflammation.

It is well recognized the patients with UC vary in the extent to which the colon is affected, the phenotype varying from rectal involvement only to pan-colitis. We have previously shown that gut microbiota do not differ based on anatomical sites of the colon in patients with UC or healthy controls (206). However, there are relatively few studies that have investigated the compositional differences in the gut microbiota between inflamed and non-inflamed mucosal sites of the colon in UC (207). In the present study, we aimed to explore whether the profiles

of gut dysbiosis differ based on mucosal inflammatory states in patients with limited extent ulcerative colitis. In addition, we used machine learning algorithms to delineate patterns of gut microbial clustering to identify key functional communities.

3.3 Results

Patients with ulcerative colitis limited to proctosigmoid or left colon, attending outpatient elective colonoscopy at the University Hospital Birmingham for assessment of their disease activity were recruited into this study. Clinical metadata and disease activity scores (Mayo endoscopic subscore) were obtained. Colon biopsies were taken from distal sites of inflamed tissue (sigmoid or rectum) and proximal sites of endoscopically non-inflamed tissue (descending colon). Presence and absence of inflammation at these sites was further confirmed by paired histological analysis by an independent histopathologist. Histology was not scored therefore not recorded. Faecal calprotectin analysis was not done as part of this study. Ethical approval was obtained by the University of Birmingham Human Biomaterials Resource Centre for this study. As this was an exploratory pilot study, no formal power calculation was done.

3.3.1 Clinical characteristics

15 patients were recruited into the study. Demographics are shown in Table 1. Inflammation was limited to rectosigmoid in 5 patients and up to hepatic flexure in 10 patients. Mayo 3 endoscopic subscore was present in 4 patients, Mayo 2 in 4 patients and Mayo 1 in the remaining 7 patients. All patients were on aminosalicylates and 6 were on concurrent immunomodulatory therapy.

Table 3 - 1 : Demographics and clinical characteristics of participants.

Age (mean years)	42.1
Gender (F:M)	9:6
Disease extent	
Proctosigmoiditis	9/15 (60%)
Left sided colitis	6/15 (40%)
Medications	
Mesalazine	15/15 (100%)
Immunomodulators	5/15 (33.3%)
Endoscopic disease score	
Mayo 1	7/15(46.7%)
Mayo 2	4/15 (26.7%)
Mayo 3	4/15 (26.7%)

3.3.2 Quality control of demultiplexed FASTQ files

As shown in Figure 3 - 1, a total of 4,028,486 sequences were recovered with a median sequencing depth of 139,147 per sample. After trimming, filtering and binning a total of 2,187,370 ASV's were retained with a median of 77,149 per sample (Figure 3 - 1).

Minimum:	83715
Median:	139147.0
Mean:	134282.866667
Maximum:	202876
Total:	4028486

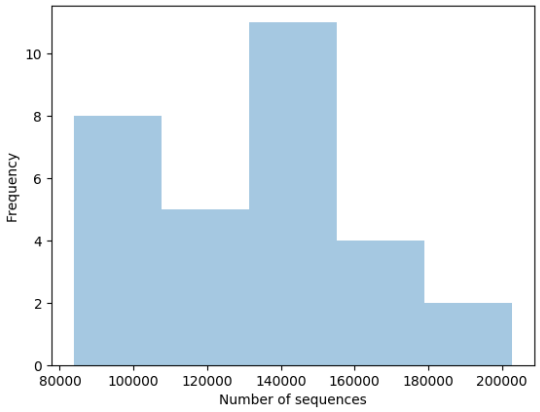


Figure 3 - 1 : Demultiplexed sequence counts summary

A total of 4,028,486 sequences were recovered with a median sequencing depth of 139,147 per sample.

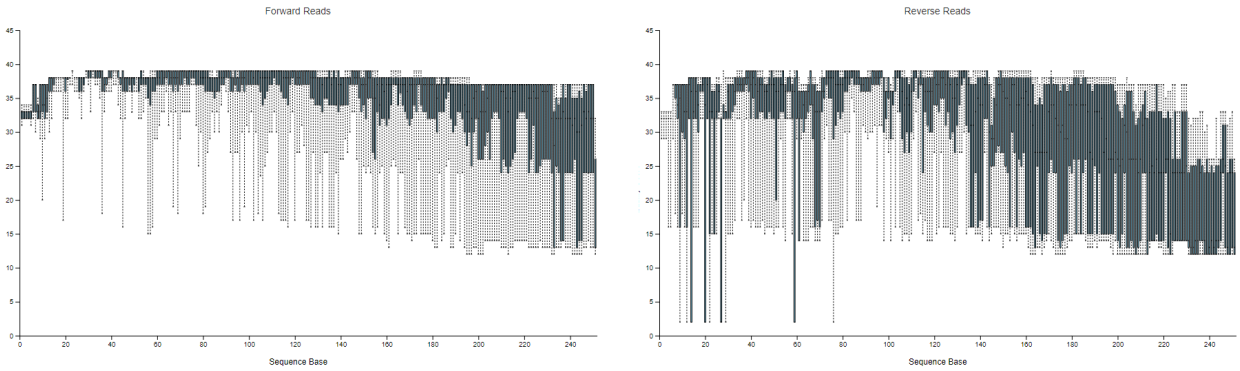


Figure 3 - 2 : QC plot for filtering and trimming of reads

Visualisation of QC plot generated using a random sampling of 10000 out of 4028486 sequences without replacement. Y-axis denotes Phred quality scores (Q scores) and X-axis denotes sequence base number. Forward reads were trimmed at position 13 and truncated to position 232. Reverse reads were trimmed at position 28 and truncated to position 159. As V4 region of the 16S gene is 254bp there would be sufficient overlap in forward and reverse reads for alignment.

3.3.3 Diversity analysis

Alpha diversity

There were no differences in paired microbial diversity between inflamed and uninflamed segments of the colon as measured by 3 metrics: Chao1 (richness), the Shannon index (evenness) and Faith's phylogenetic diversity. Alpha diversity index plots are shown in Figure 3 - 3. The alpha diversity did not change with severity of inflammation, medications or distribution of disease.

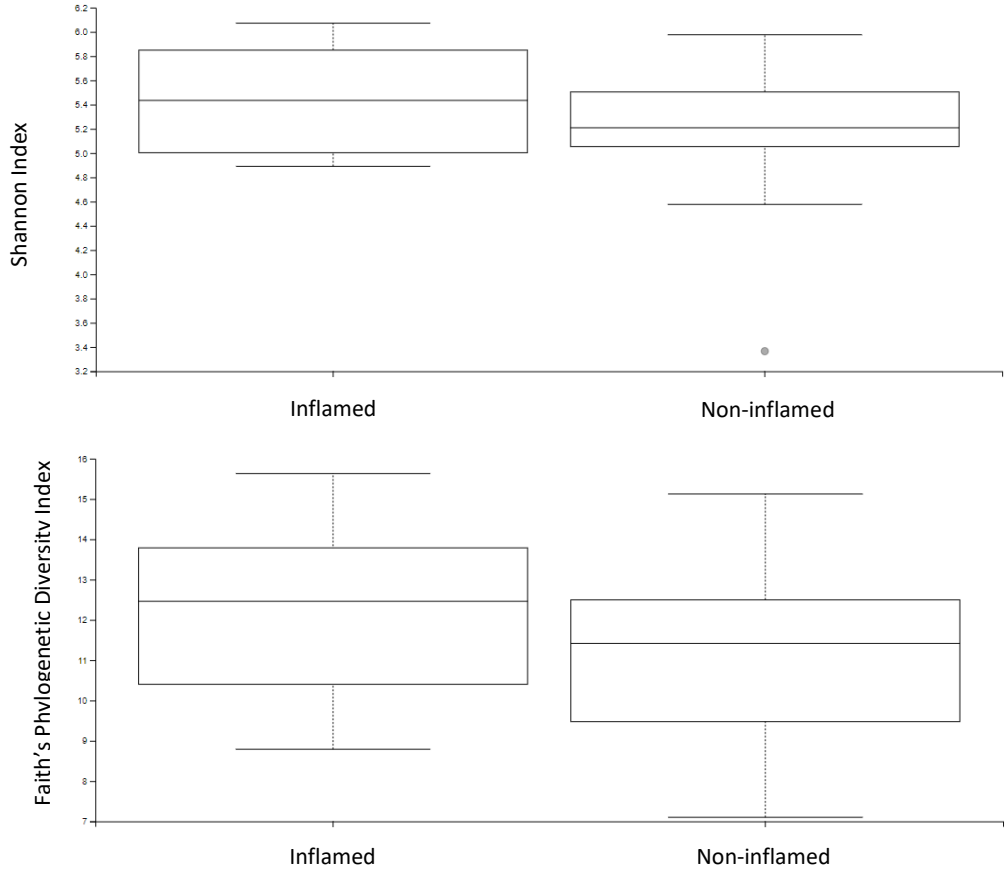


Figure 3 - 3: Alpha diversity of gut microbiota between inflamed and non-inflamed tissue

Alpha diversity as estimated by Shannon and Faith's diversity index analysis demonstrated no difference in richness and diversity between inflamed and non-inflamed tissue ($p = 0.47$ and 0.27 respectively).

Beta diversity

Paired assessment by PERMANOVA revealed that the global microbial composition (beta diversity) between inflamed and non-inflamed segments were very similar as shown in Figure 3 - 4. These similarities when visualised through non-metric multidimensional scaling (NMDS) Bray-Curtis dissimilarity revealed that among almost all patients there was very little variation in the microbial composition between these two sites (Figure 3 - 5). These findings were further confirmed using principal coordinates analysis plots of weighted and unweighted UniFrac. These similarities in beta diversity were observed regardless of Mayo endoscopic grading of inflammation, medications or distribution of disease.

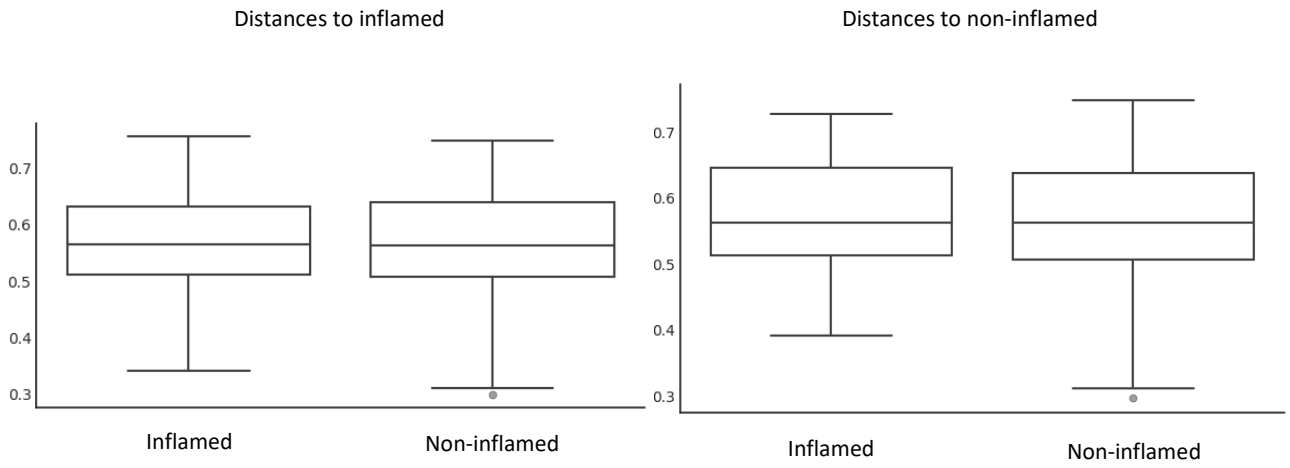


Figure 3 - 4 : Beta diversity of gut microbiota between inflamed and non-inflamed tissue

Pairwise beta diversity as estimated unweighted unifrac diversity index analysis demonstrated no difference in global microbial composition inflamed and non-inflamed tissue ($p = 0.82$).

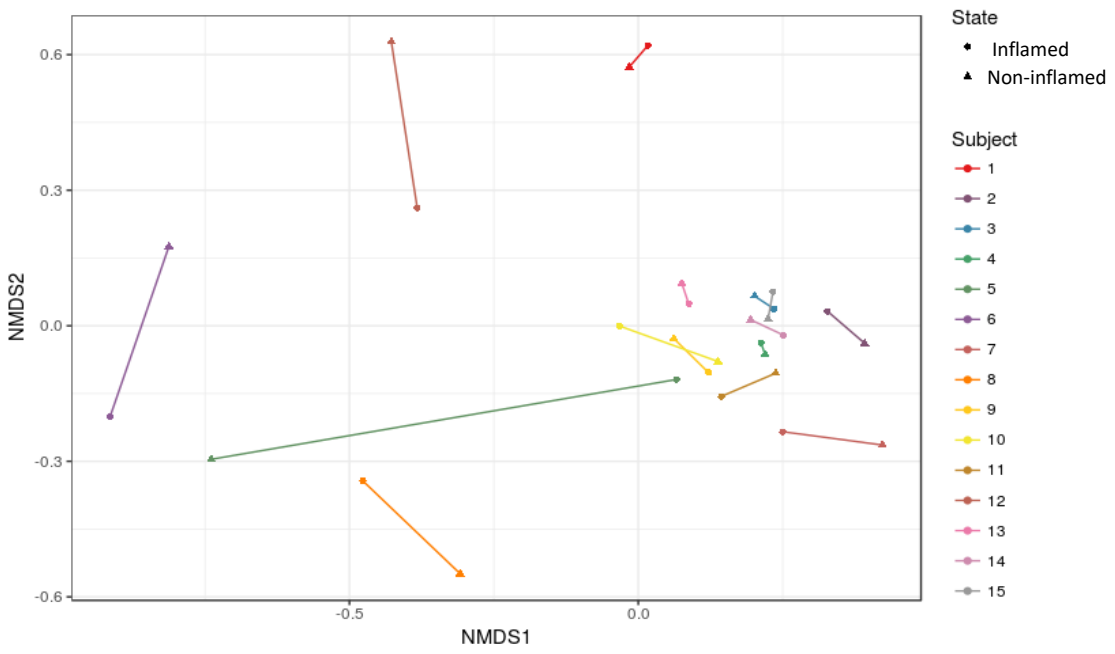


Figure 3 - 5 : NMDS plot of Bray-Curtis dissimilarity between inflamed and non-inflamed tissue

Bray-Curtis dissimilarity plot demonstrates that microbial composition appears to be largely similar between inflamed and non-inflamed tissue. No difference or specific patterns were observed based on of Mayo endoscopic grading of inflammation, medications or distribution of disease.

3.3.4 Microbial characterisation

A total of 543 OTUs were identified after filtering. Dominant phyla across all samples included *Firmicutes* (59%), *Bacteroidetes* (23%), *Proteobacteria* (11%) and *Actinobacteria* (7%). Phylum and genus level abundances are shown in Figure 3 - 6.

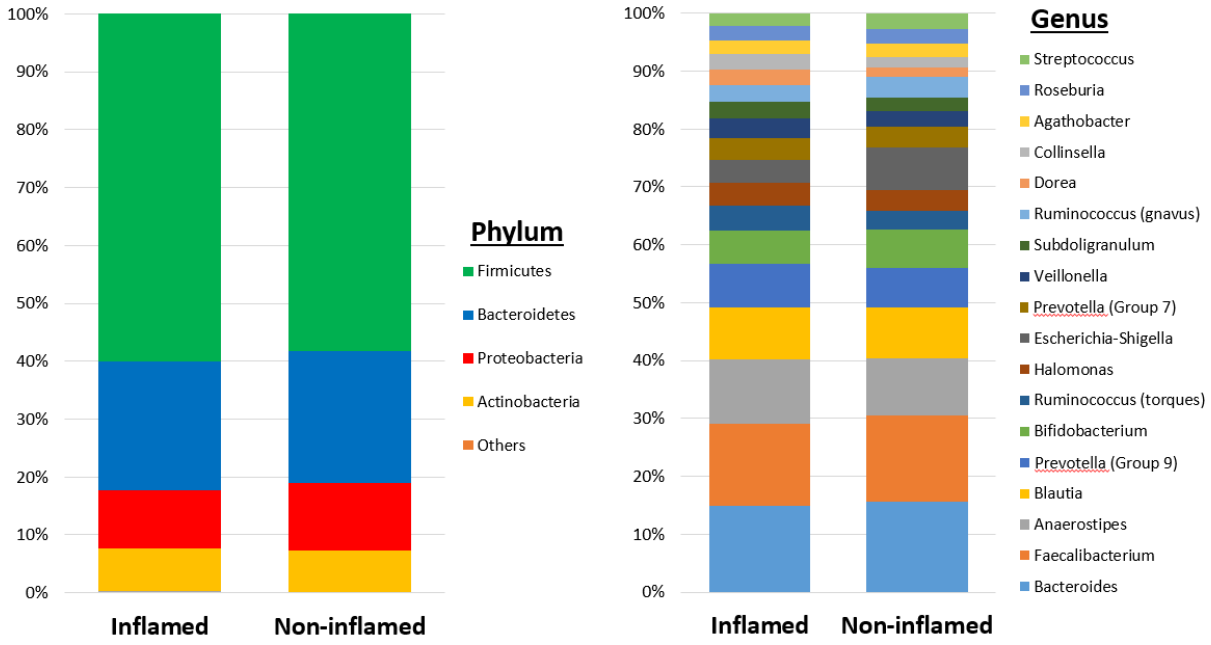


Figure 3 - 6 :Taxonomic classification at phylum and genus level

Bar plot demonstrating phylum and genus level differences between inflamed and non-inflamed sites. Dominant phyla across all samples included Firmicutes (59%), Bacteroidetes (23%), Proteobacteria (11%) and Actinobacteria (7%). No significant differences in phylum were seen between the two sites.

3.3.5 Differential taxonomic analysis

DESeq2 analysis demonstrated two significant findings amongst OTUs: *Clostridiaceae* were less abundant (log2FC = 5.6, p = 0.049) and *Atopobiaceae* (log2FC=7.78, p = 0.005) significantly more abundant taxonomic groups at inflamed sites of the colon. No significant differences were identified in OTUs at other taxonomic levels between sites of inflamed and non-inflamed tissue.

No significant association was identified between OTUs and grade of endoscopic scoring of inflammation, disease extent or concurrent therapy with immunomodulators.

A trend was observed towards increases in taxa belonging to Firmicutes, Clostridiales in non-inflamed tissues compared to inflamed as shown in Figure 3 - 7. Individual differences in these taxa were however were not shown to be significant.

Figure 3 - 7 : DESeq2 analysis showing trends in changes of SCFA producing bacteria

A trend was observed towards increases in taxa belonging to Firmicutes and Clostridiales in non-inflamed tissues compared to inflamed sites. Individual differences in these taxa were however were not shown to be significant.

3.3.6 Predicted functional characterisation of gut microbial communities

PICRUSt functional inferences of the microbiota (Figure 3 - 8) revealed a significantly higher proportion of genes were associated with bacterial motility (p=0.017), bacterial chemotaxis (p=0.004) and flagellar assembly (p=0.019).

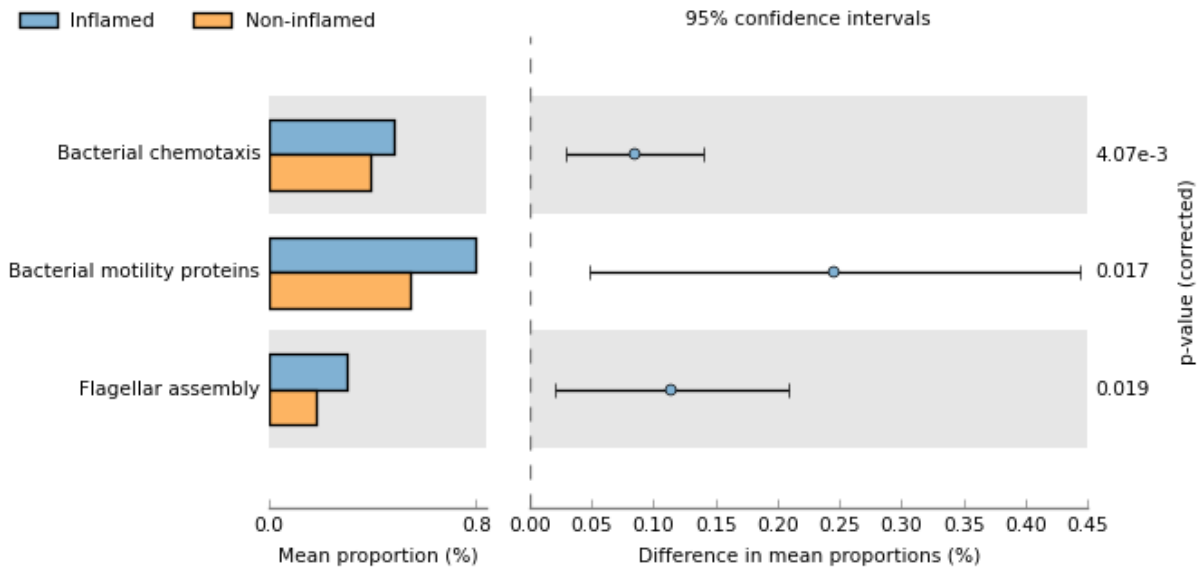


Figure 3 - 8 : Differences in predicted function of gut microbiota between inflamed and non-inflamed mucosa using PICRUST

Significantly higher proportion of genes were associated with bacterial motility (p=0.017), bacterial chemotaxis (p=0.004) and flagellar assembly (p=0.019) were seen at inflamed sites compared to non-inflamed sites.

3.4 Discussion

Our results highlight taxonomic and potential functional changes at sites of colonic mucosal inflammation in patients with ulcerative colitis. We found that the *Clostridiaceae* family were significantly underrepresented at inflamed sites. These gram-positive, rod-shaped bacteria play a crucial role in regulation of metabolic and immune processes and are strongly involved in the maintenance of gut homeostasis (104, 208). *Clostridiaceae* perform their metabolic functions through the production of short-chain fatty acids (SCFAs) generated via the fermentation of dietary fibers. SCFAs are essential as nutrients for colonocytes and regulate proliferation and gene expression. Butyrate limits intestinal inflammation by promoting the maturation of naïve CD4 T cells towards a population of adaptive immune cells that suppress inflammatory responses known as regulatory T cells (Tregs) as well as reduction in pro-inflammatory cytokines production by macrophages (103, 209, 210). In keeping with this finding, we observed that rather than significant alterations in individual bacterial species, colonic inflammation was associated with shifts in certain ecological communities. Inflammation was associated with a reduction in SCFA producing bacterial species within the *Faecalibacterium, Bifidobacterium*, *Ruminococcus, Ruminiclostridium, Paraprevotella, Parabacteroides, Bifidobacterium, Lachnospiraceae and Clostridiales* taxonomic groups. Taken together these findings suggest that reduction in populations of gut microbial SCFA producers could contribute to or trigger immune mediated colonic inflammatory responses associated with ulcerative colitis. These outcomes were consistent regardless of the Mayo endoscopic sub-score, disease extent and use of immunomodulatory medication.

Therapeutic use of butyrate in the form of butyrate enemas has been explored in clinical studies in ulcerative colitis with early uncontrolled studies from the 1990s showing promising results (211). Butyrate enemas have shown to decrease nuclear translocation of NF-κB in colonic macrophages in patients with distal UC patients (212). Different approaches,

including enemas of butyrate and/or mixtures of SCFAs (acetate, propionate, and butyrate) have resulted in diverse clinical outcomes (213). Both butyrate or mixtures of SCFAs in enemas have demonstrated different degrees of clinical and histological improvement in active UC patients and diversion colitis (colitis as a consequence of faecal diversion in patients with an ileostomy) (214). SCFAs enemas (100 ml of 80 mM acetate, 30 mM propionate, and 40 mM butyrate twice a day for 6 weeks) was able to produce clinical remission only in a subset of UC patients (215). Moreover, butyrate enemas (60 ml of 100 mM once daily for 20 days) do not affect daily symptoms score, stool consistency and frequency in patients with UC. This lack of success with butyrate however may possibly be due to limitations associated with adequate formulation and delivery of SCFA compounds into the colon.

Bacterial functions as predicted based on 16S rRNA gene profile indicated that bacteria at sites of inflammation had a significantly higher proportion of genes associated with motility, chemotaxis and flagellar assembly. Motility is closely linked with chemotaxis and facilitates the ability of bacteria to orientate along certain chemical gradients (216). The flagellum is the best-known mode of motility in numerous pathogenic and non-pathogenic bacteria and plays an important role in adhesion to and invasion into host cells bacteria (217, 218). Several flagellin proteins have been shown to have immunomodulatory properties and play a significant role in innate immunity and as a dominant antigen of the adaptive immune response. Expression of proinflammatory cytokines such as IL-8 are elicited by flagellins of *Escherichia coli*, *Pseudomonas aeruginosa* and *Clostridium difficile* in intestinal epithelial cell cultures (219, 220). These findings suggest that bacteria at sites of inflammation are more likely to possess abilities to invade colonic mucosal surfaces and initiate pro-inflammatory immune responses.

In this study, we used predictive modelling to minimise any prior assumptions on the data set like linearity or normal distribution of the data sets. Through hierarchical clustering, we could identify group of OTUs that might be involved in the biological process. The Random Forest analysis resulted in a high error which suggested that the microbial differences between inflamed and non-inflamed sites were not significantly heterogeneous.

Previous studies exploring mucosal microbial profiles in UC have been cross sectional and have not established differences in dysbiosis in relation to inflammatory activity. Our study demonstrates a reduced abundance of immuno-regulatory microbial community at sites of mucosal inflammation and attempts to construct causative mechanisms in host-microbiota relationships. The reason for these inflammation-specific differences and the phenomenon of limited extent / segmental colitis in UC can be postulated to be a consequence of differences in oxygen gradients, also known as the ‘oxygen hypothesis’. Here microvascular changes in dominant blood supply result in shifts of bacterial communities from obligate to facultative anaerobes resulting in a disruption in anaerobiosis and points to a role for oxygen in gut dysbiosis (221). Furthermore, an increase in the oxygen concentration could be caused by hyperaemia of the gastrointestinal tract during chronic inflammation, results in the release of hemoglobin carrying oxygen in the colonic mucosa and lumen thereby promoting a state of dysbiosis.

The relatively small cohort of 15 patients and lack of a comparator healthy control population is likely to be a significant limiting factor in this study. This possibly may have led to failure to identify differences in abundances of other crucial gut commensals in regulation or contribution to inflammatory pathways. An additional limitation is, similar to other metataxonomic and metagenomic approaches, our study only demonstrates differences in potential functional mechanisms. Further larger cohort studies are needed in order to explore transcriptional and metatranscriptional differences at sites of mucosal inflammation. This

would consequently help uncover the relationship between dysbiosis and inflammation in IBD towards understanding the cause and effect paradigm in IBD.

3.5 Acknowledgements

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CHAPTER 4

Th17 cells dominate the colonic mucosal immune response in primary sclerosing cholangitis associated inflammatory bowel disease

4.1 Abstract

Introduction: Primary sclerosing cholangitis (PSC) is an idiopathic chronic cholestatic liver disease associated with ulcerative colitis (UC). PSC is thought to be a consequence of a genetically predisposition, dysregulated immune response and unknown factors including the gut microbiome. The colonic mucosal immune response in PSC associated colitis (PSC-IBD), however, has been poorly defined. In this study, we analysed the characteristics of colonic mucosal CD4 T cells in patients with PSC-IBD.

Methods: Colon biopsies were collected from patients with PSC-IBD (n=14), UC (n=23) and healthy controls (n=21). Lamina propria mononuclear cells were extracted and analysed by flow cytometry.

Results: PSC-IBD and UC were characterised by a significantly higher frequency of colonic mucosal CCR6+CD161+ Th17 cells compared to healthy controls (17.5% vs 11.1%; p=0.006 and 18.62% vs 11.1%; p=0.009 respectively). CCR6-CXCR3+CCR5+ Th1 cells were significantly lower in PSC-IBD and UC compared to healthy controls (15.58% vs 24.06%; p=0.02 and 15.74% vs 24.06%; p=0.01 respectively). Significantly increased frequencies of IL17 producing CD4 cells were observed in both PSC-IBD and UC compared to controls ((7.67% vs 4.7%; p<0.001 and 10.39% vs 4.70%; p=0.001 respectively). CD127-CD25+FoxP3+ T regulatory cell frequencies was significant elevated (p<0.001) and CCR6-CCR5-CXCR3- Th2-like cell frequencies were reduced only in UC and not PSC-IBD when compared to controls (p=0.03). Although there were no differences in TNFα and IFNγ producing CD4 cells, patients with PSC-IBD and UC had a significantly higher frequency of IL17/IFNγ dual producing CD4 cells (3.12% vs 1.76% respectively; p=0.005, 2.77% vs 1.76% respectively; p=0.02). Correlation analysis of PSC-IBD and controls demonstrated that Th17 frequencies positively correlated with increasing frequencies of T regulatory cells and negatively with Th1 (p<0.05). No significant associations were identified between these CD4 subsets and Mayo endoscopic subscore and disease distribution.

Conclusions: Our study demonstrates for the first time that the colonic mucosal immune response in PSC-IBD is characterised by significantly higher Th17 cells and lower Th1 cells compared to controls. Patients with PSC-IBD have higher IL-17 and IL17/IFNγ dual producing CD4 cells. Our findings highlight the need to explore the role of key players such as the gut microbiome and bile acids in mucosal T cell homeostasis and Th1/Th17 plasticity in PSC-IBD.

4.2 Introduction

Primary sclerosing cholangitis (PSC) is a chronic liver disease that is characterised by progressive inflammation and fibrotic stricturing of the biliary tree (222). Its pathogenesis is poorly understood and is presumed to reflect a complex multifactorial interaction between immune dysregulation, gut microbial dysbiosis and possibly changes in bile acid homeostasis in a genetically at risk individuals (223).

PSC is highly comorbid with inflammatory bowel disease (IBD), which is ultimately diagnosed in approximately 75% of patients primarily in a clinical form of ulcerative colitis (UC). The colonic inflammation in PSC-IBD however behaves differently to conventional UC without PSC (224).[3] Patients with PSC-IBD have a milder but more extensive form of colonic inflammation (pancolitis) with rectal sparing. Furthermore, along with the lack of overlap in the genome wide association studies (GWAS) analyses significant distinctions have also been demonstrated in gut microbial profiles between patients with PSC-IBD and UC (23, 206, 225, 226). These findings have collectively strengthened the argument that PSC-IBD represents a separate disease entity with potentially different triggers and immune mechanisms responsible for colonic inflammation. As the colonic mucosal immunological changes remain largely unexplored in PSC-IBD we undertook a study to characterise the differences in the colonic mucosal immune profiles in patients with PSC-IBD, UC and controls.

4.3 Results

4.3.1 Study subjects

All patients booked for a colonoscopy as part of their clinical care were recruited from outpatient clinics at the University Hospitals Birmingham. Patients with PSC-IBD were selected based on a prior radiological or histological confirmed diagnosis of large-duct PSC. Both PSC-IBD and UC patients also had a prior confirmed endoscopic and histological diagnosis of UC. Patients with no prior known co-morbidities being referred for investigation of rectal bleeding and either a normal lower GI endoscopy or a diagnosis of haemorrhoids were selected as healthy controls for this study. Colonic biopsies were collected from the sigmoid colon for all patients. Consent for this study was obtained through the HBRC biobank ethics. Blood samples collected in outpatients clinics for PSC-IBD patients immediately before their colonoscopy were used as part of multivariate analysis in this study.

4.3.2 Patient characteristics

Fifty-eight patients were recruited into this study – 21 healthy controls (HC), 14 patients with PSC-IBD and 23 patients with UC. There was no significant difference in the age or gender between the three groups. Patients with PSC-IBD and UC had no significant difference in distribution, endoscopic severity of colitis and medication use. Three patients with PSC-IBD were post liver transplant, two had cirrhosis (Child Pugh A) and five were on ursodeoxycholic acid. Detailed demographics are shown in Table 1.

Table 4 - 1 : Demographic data of the three cohorts in this study

	HC (n=21)	PSC-IBD (n=14)	UC (n=23)
Age (mean)	40.2 years	39.5 years	39.6 years
Gender M:F	12:9	9:5	12:11
Ulcerative colitis characteristics			
Pan UC		14/14 (100%)	18/23 (78%)
Mayo endoscopic subscore			
0		11/14 (79%)	17/23 (74%)
1		0/14 (0%)	0/23 (0%)
2		1/14 (7%)	2/23 (9%)
3		2/14 (14%)	4/23 (18%)
Drugs			
Mesalazine		13/14 (93%)	21/23 (91%)
Azathioprine		8/14 (57%)	15/23 (65%)
Vedolizumab		3/14 (21%)	4/23 (17%)
Other biologics		2/14 (14%)	4/23 (17%)

4.3.3 Lamina propria mononuclear cell immunophenotyping

Th17 cells dominate colonic mucosal immunophenotype in PSC-IBD and UC compared to healthy controls

PSC-IBD and UC were characterised by a significantly higher frequency of colonic mucosal CCR6+CD161+ Th17 cells compared to controls (17.5% vs 11.1%; p=0.006 and 18.62% vs 11.1%; p=0.009 respectively) - Figure 4 - 1. The Th17 frequencies were similar between PSC-IBD and UC.

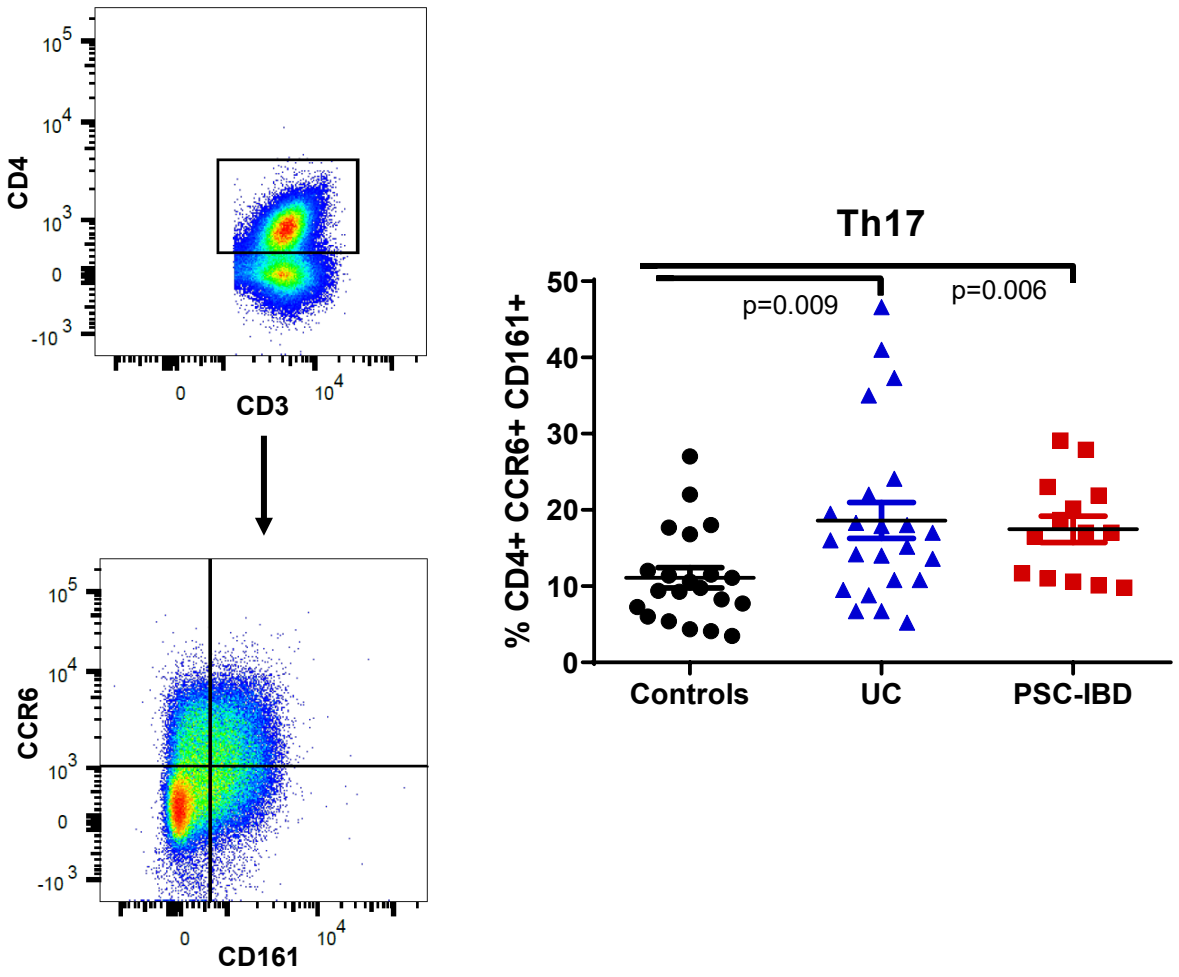


Figure 4 - 1 : Differences in Th17 between cohorts

PSC-IBD and UC were characterised by a significantly higher frequency of colonic mucosal Th17 cells compared to controls

Regulatory T cells are paradoxically elevated in UC compared to healthy controls

The frequency of regulatory T cells CD127-CD25+FoxP3+ were significantly elevated in UC compared to healthy controls (8.93 % vs 4.43%; p<0.0001) - Figure 4 - 2. Similar trend was seen in PSC-IBD when compared healthy controls however this did not reach statistical significance (6.56% vs 4.43%; p=0.07).

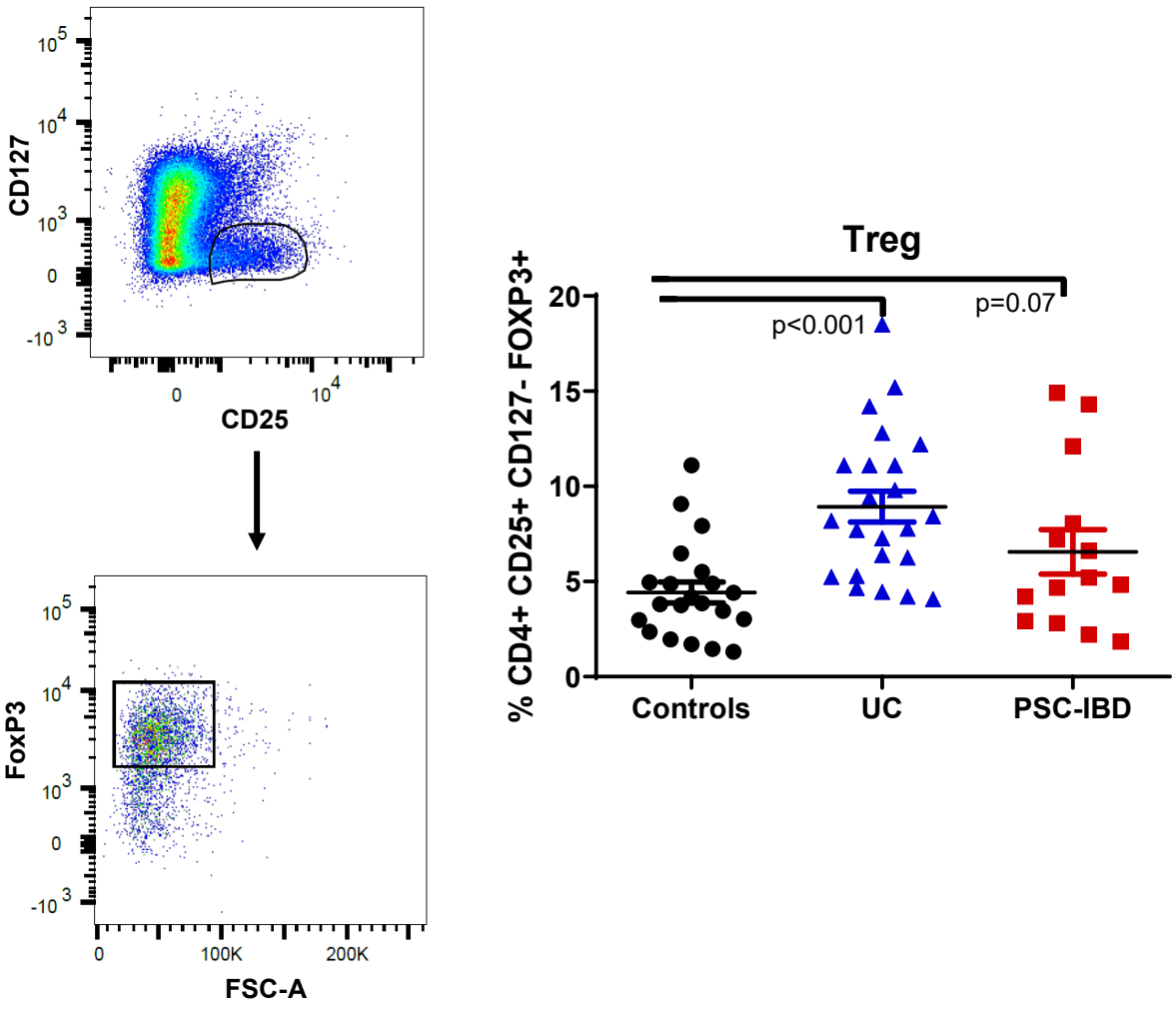


Figure 4 - 2 : Differences in Tregs between cohorts

PSC-IBD and UC were characterised by a significantly higher frequency of colonic mucosal Treg cells compared to controls

Th1 (like) cell subtype

Frequencies of CCR6-CD161-CXCR3+CCR5+ Th1 cells were significantly reduced in both PSC-IBD and UC in comparison to healthy controls (15.58% vs 24.06%; p=0.02 and 15.74% vs 24.06%; p=0.01 respectively) - Figure 4 - 3.

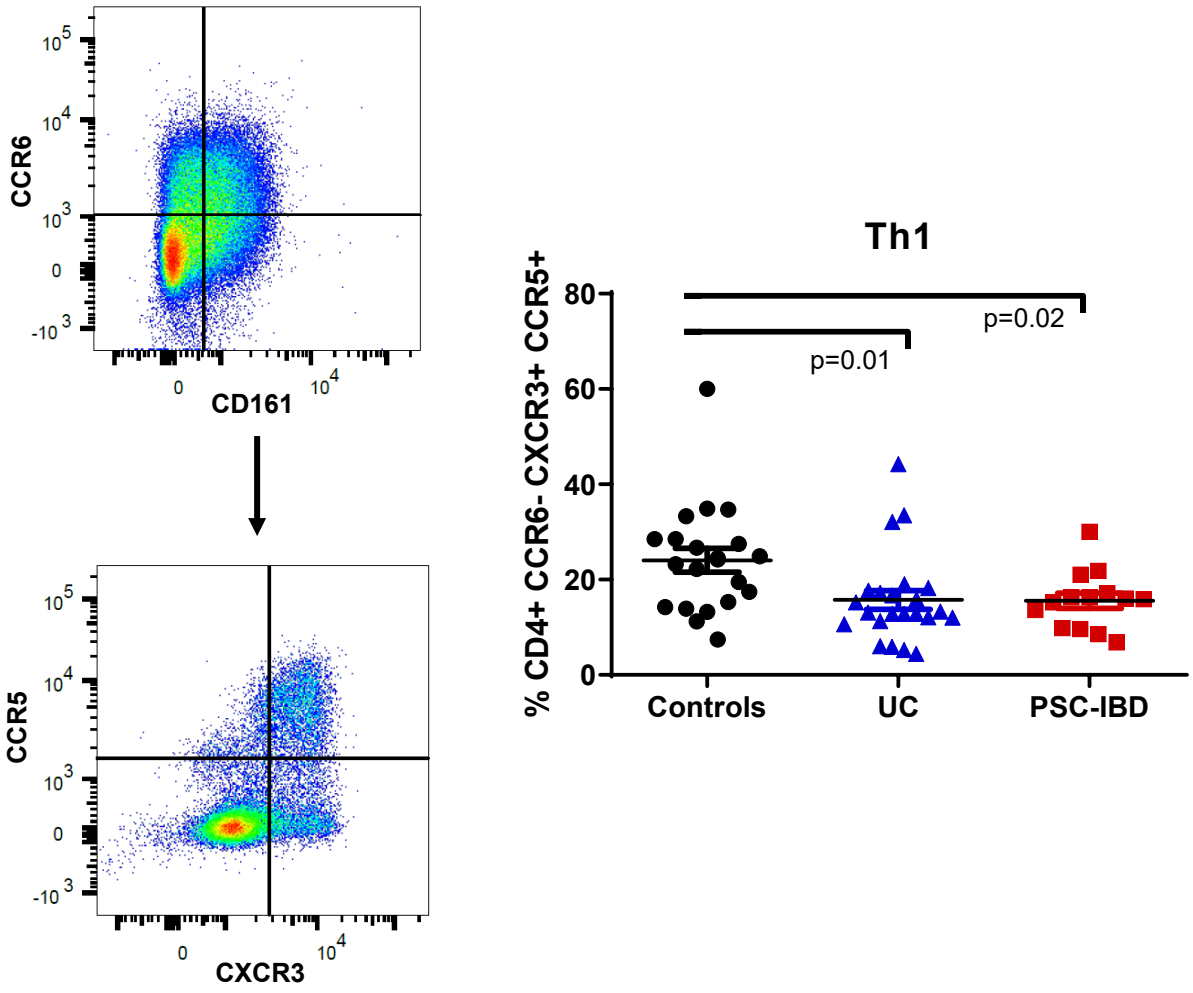


Figure 4 - 3 : Differences in Th1 like cells between cohorts

PSC-IBD and UC were characterised by a significantly lower frequency of colonic mucosal Th1 cells compared to controls

Th2 (like) cell subtype

The Th2-like population as defined by CCR6-CD161-CXCR3-CCR5- CD4 cells were significantly higher in UC but not in PSC-IBD when compared to healthy controls (15.25% vs 8.74%; p=0.03) - Figure 4 - 4. There were no differences in any of the CD4 subsets between PSC-IBD and UC.

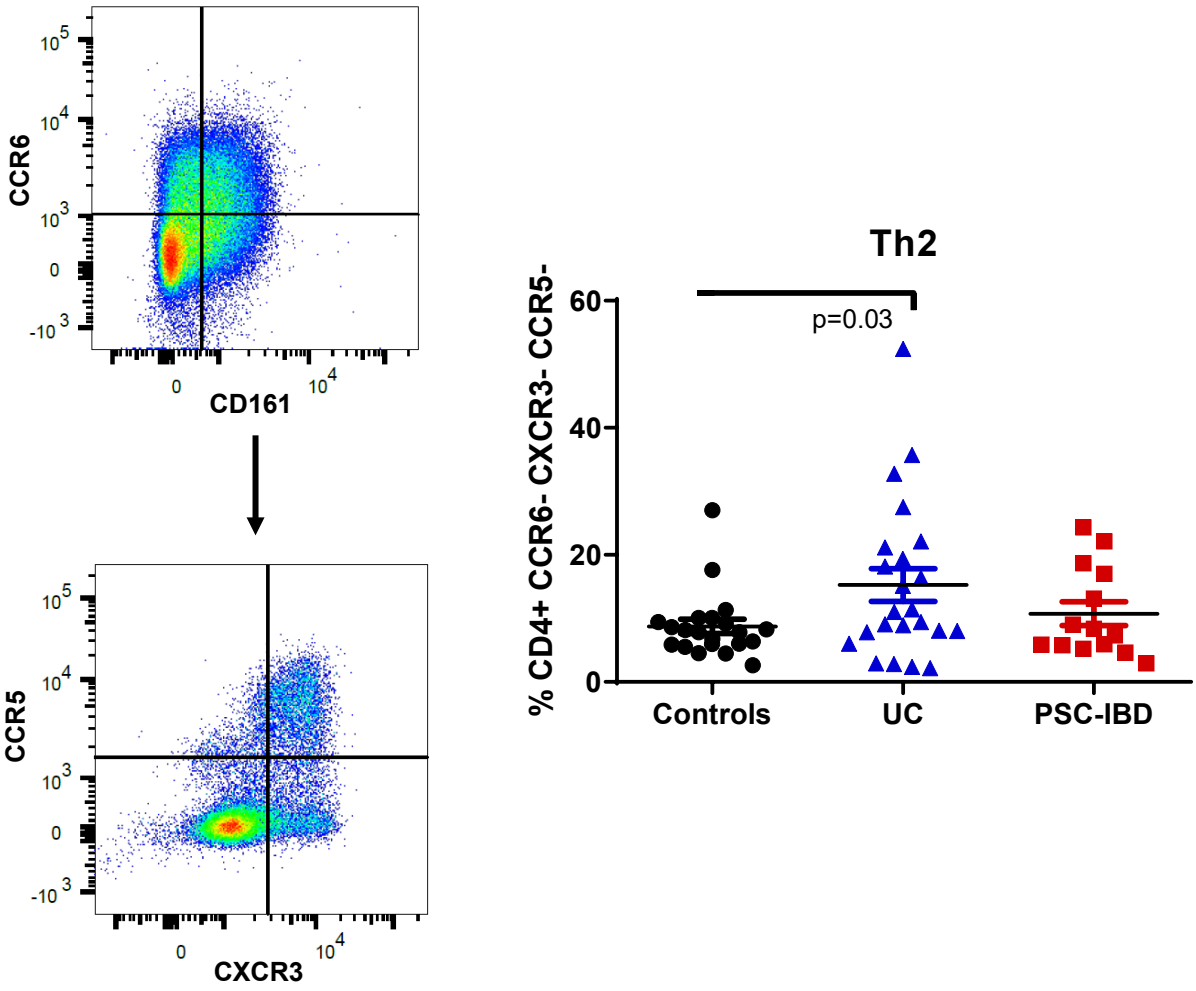


Figure 4 - 4 : Differences in Th2 like cells between cohorts

UC was characterised by a significantly higher frequency of colonic mucosal Th1 cells compared to controls

IL17 secreting CD4 T cells are significantly increased in PSC-IBD and UC patients compared to HC

Patients with PSC-IBD and UC had significantly increased frequencies of IL17 producing CD4 cells were observed in both PSC-IBD and UC compared to controls (7.67% vs 4.7%; p<0.001 and 10.39% vs 4.70%; p=0.001 respectively). These frequencies were similar between PSC-IBD and UC.

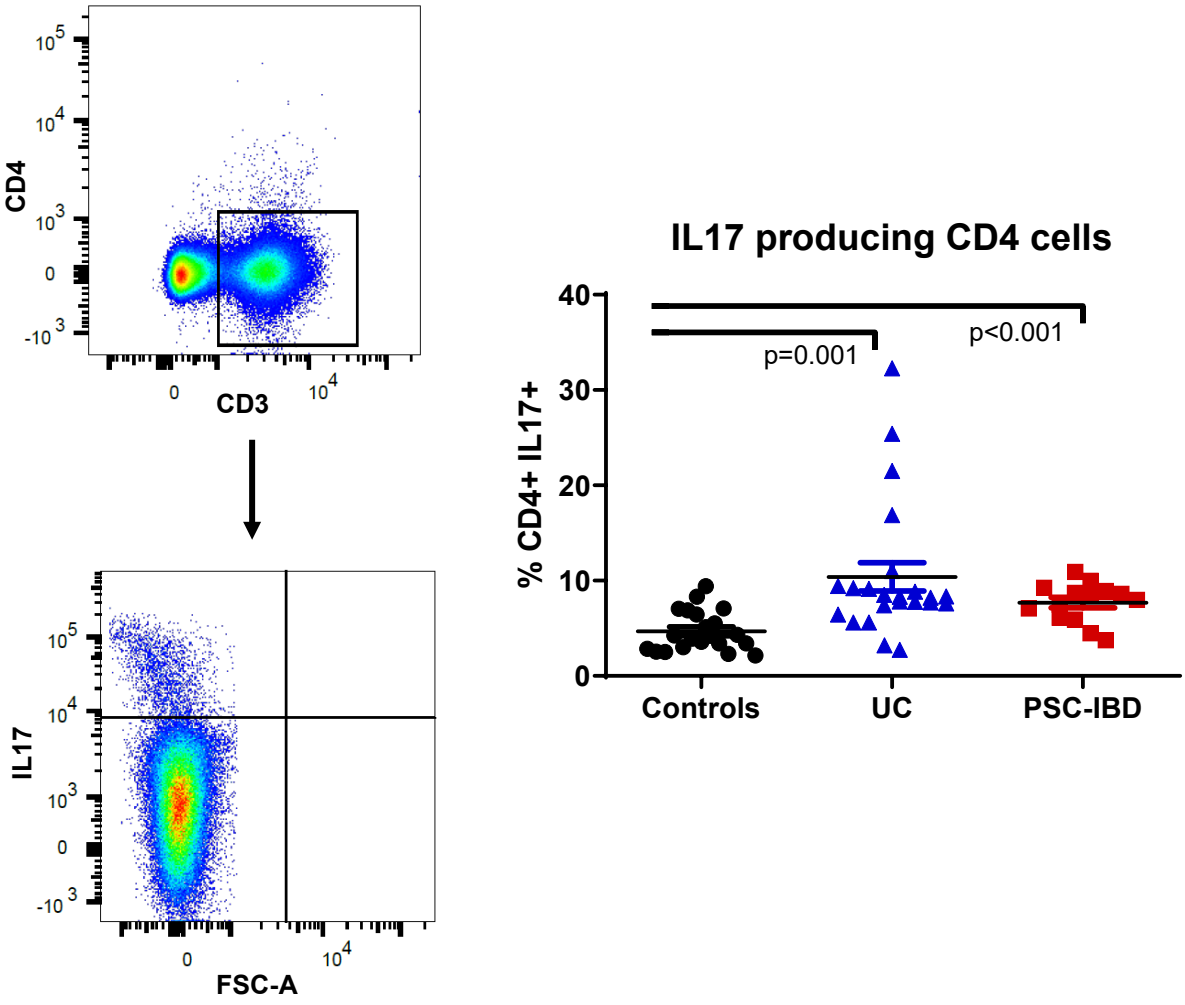


Figure 4 - 5 : Differences in IL-17 producing CD4 cells between cohorts

Consistent with Th17 cell frequencies, PSC-IBD and UC were characterised by a significantly higher frequency of colonic mucosal IL-17 producing CD4 cells compared to controls

No differences seen in IFNγ PSC-IBD and UC patients compared to HC

There were no differences in IFNγ producing CD4 cells when either of the three groups were compared to each other - Figure 4 - 6.

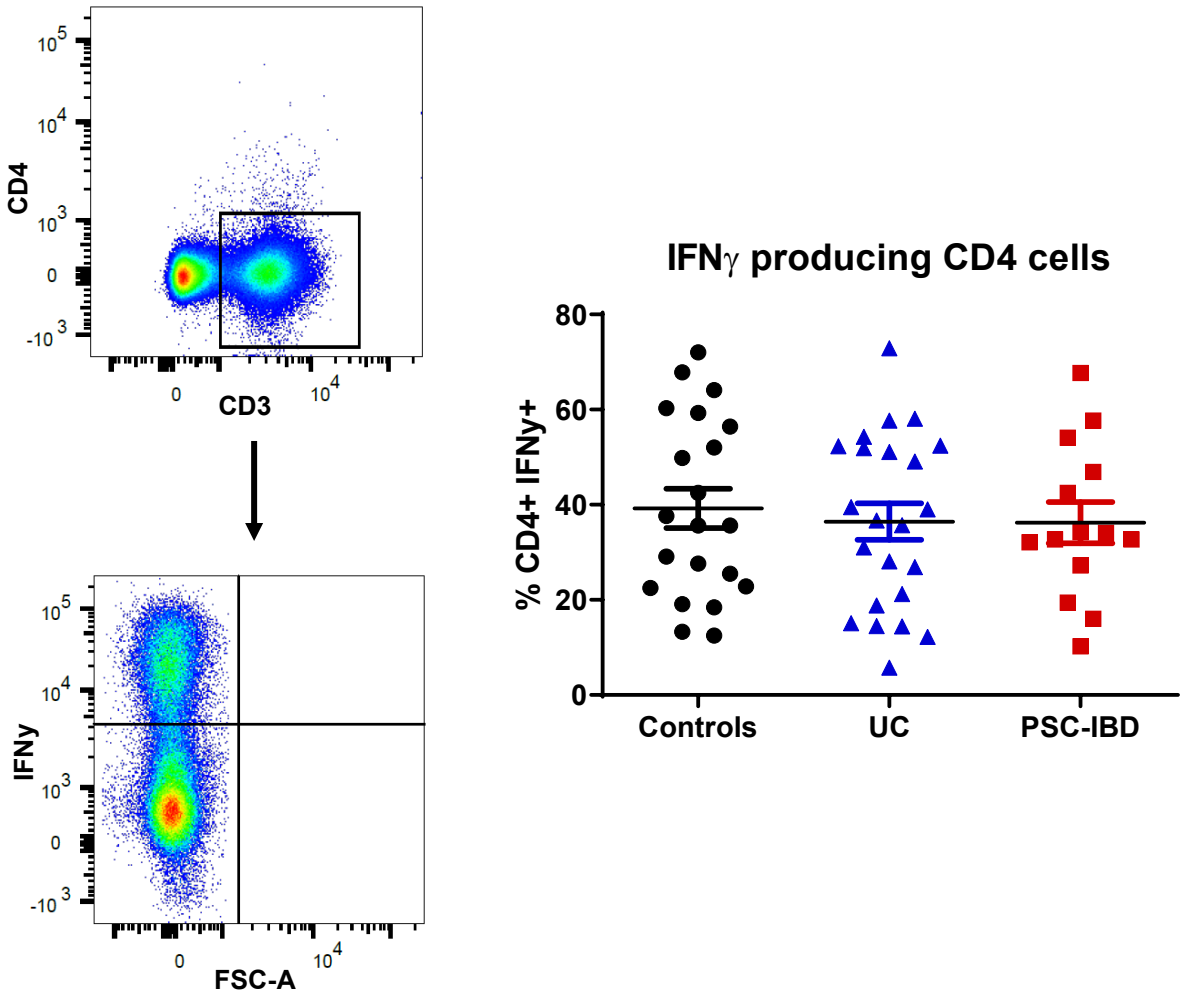


Figure 4 - 6 : Differences in IFNγ producing CD4 cells between cohorts

There were no differences in IFNγ producing CD4 cells when either of the three groups were compared to each other.

No differences seen in TNFα PSC-IBD and UC patients compared to HC

There were no differences in TNFα producing CD4 cells when either of the three groups were compared to each other (Figure 4 - 7).

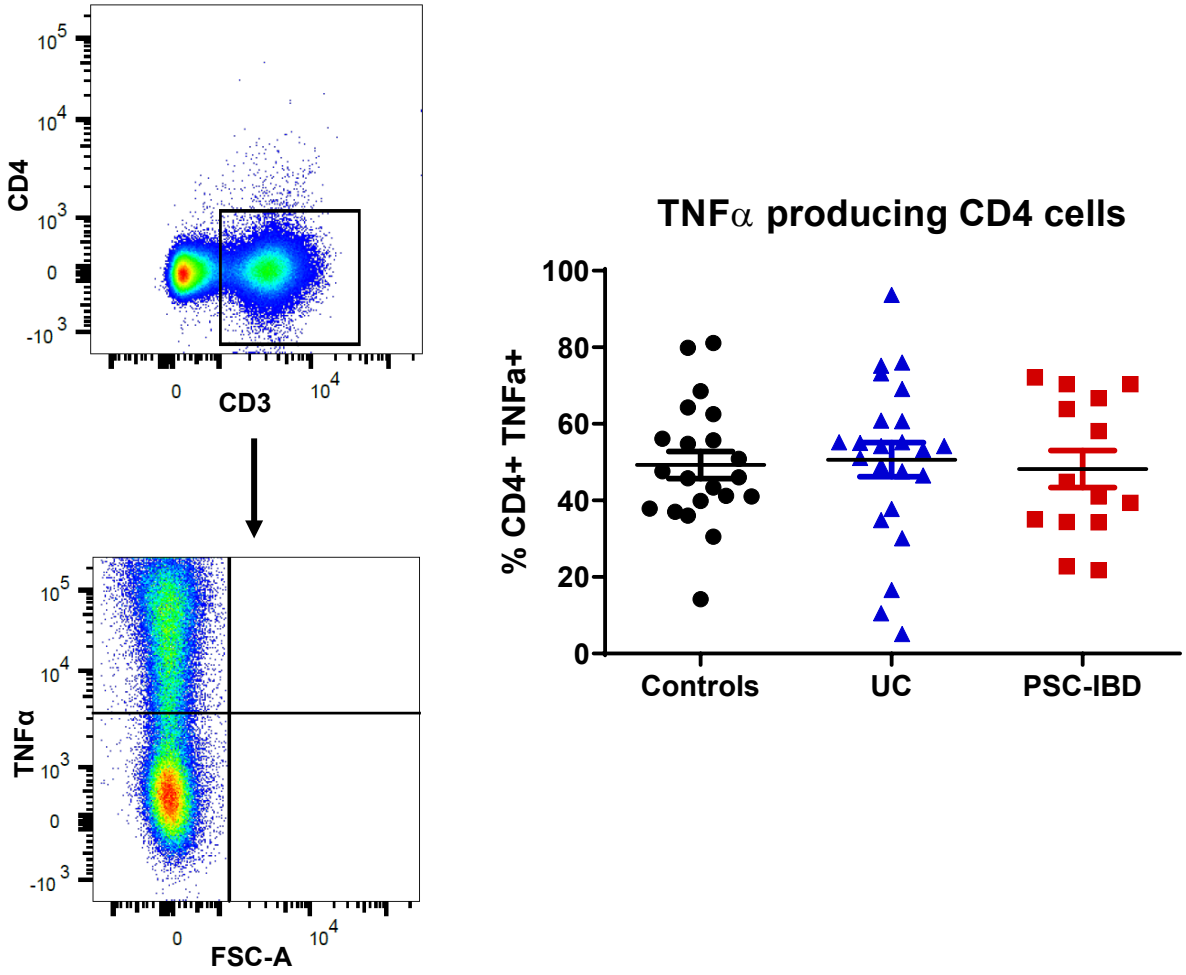


Figure 4 - 7 : Differences in TNFα producing CD4 cells between cohorts

There were no differences in TNFα producing CD4 cells when either of the three groups were compared to each other.

IL17/IFNγ dual secreting CD4 T cells are significantly increased in PSC-IBD and UC patients compared to HC

However, both PSC-IBD and UC had a significantly higher frequency of IL17/IFNγ dual producing CD4 cells compared to controls (3.12% vs 1.76% respectively; p=0.005, 2.77% vs 1.76% respectively; p=0.02) - Figure 4 - 8.

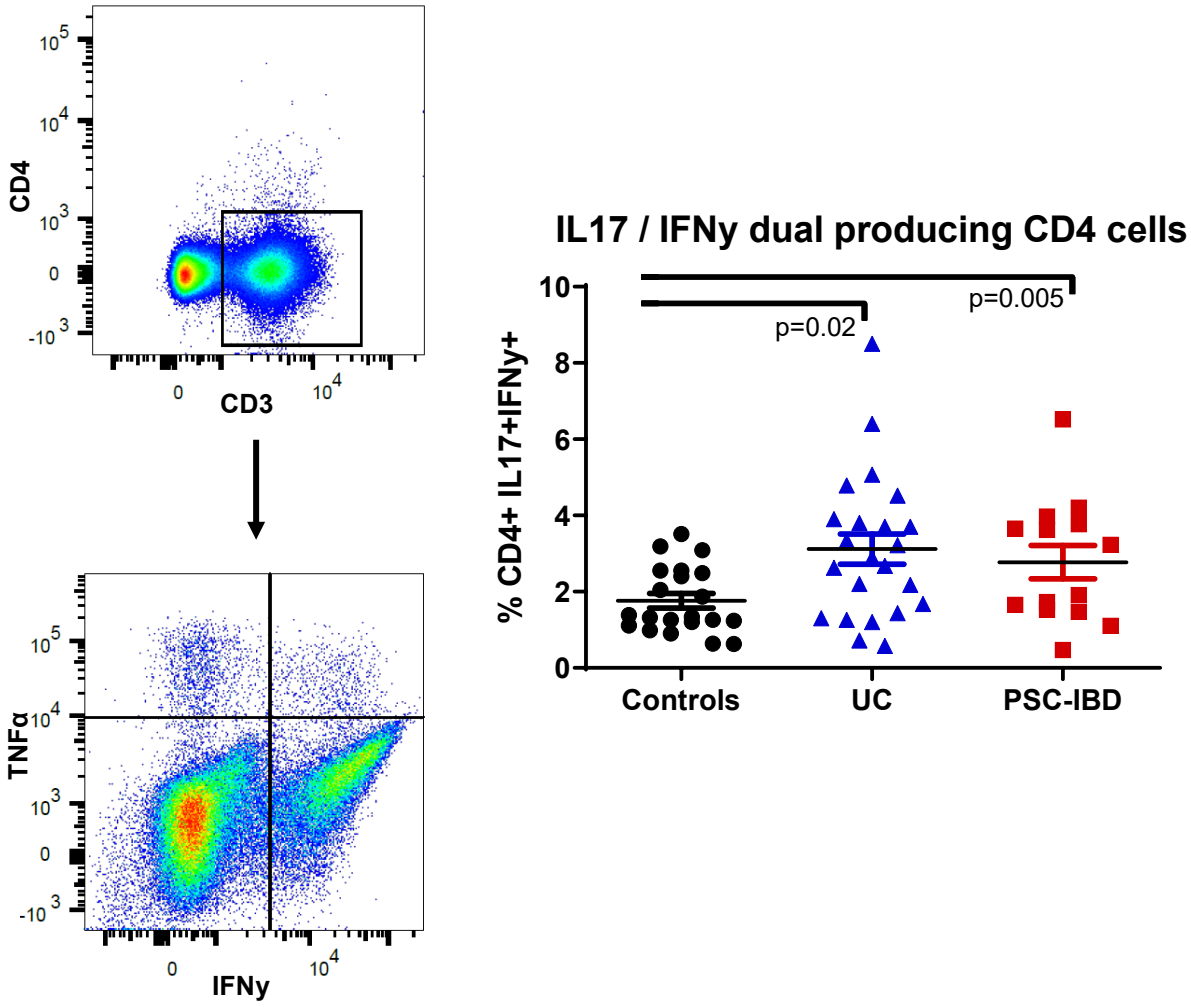


Figure 4 - 8 : Differences in IL-17 / IFNγ producing CD4 cells between cohorts

PSC-IBD and UC were characterised by a significantly higher frequency of colonic mucosal IL-17 / IFNγ producing CD4 cells compared to controls

Multivariate analysis

Multivariate analysis (linear regression) and Pearson correlation analysis demonstrated that Th17 significantly positively correlated with Tregs and negatively with Th1. Furthermore, Th17 profiles did not significantly change with disease distribution or Mayo endoscopic subscore in PSC-IBD and UC.

4.4 Discussion

The aetiopathology and mechanisms of colonic inflammation in PSC-IBD is poorly understood. Here we report the largest analysis to date of colonic mucosal immunophenotyping in PSC-IBD in comparison to UC and healthy controls. Patients with PSC-IBD and UC both have significant increases in Th17, regulatory T cells (Tregs) and IL-17 producing CD4 T cell populations compared to controls. Importantly no distinctions were seen between PSC-IBD was compared to UC.

Th17 cells are a subpopulation of $CD4^+$ T cells that are involved in the disease progression of many autoimmune and inflammatory disorders including IBD, rheumatoid arthritis and psoriasis (227-229). Th17 cells mediate their effects via the secretion of the IL-17 family cytokines - IL-17A and IL-17F along with IL-22 and granulocyte-macrophage colony-stimulating factor (GM-CSF). Multiple studies have highlighted the critical role of the Th17 response in host defence against extracellular pathogens (230). Both PSC-IBD and UC are associated with a distinct form of dysbiosis in comparison to healthy controls. Furthermore, the dysbiosis associated with both colonic mucosally adherent bacteria and faecal microbiota in PSC-IBD significantly differs to that seen in UC (156, 226). Patients with PSC-IBD have an expansion of species in phylum Bacteroides and underrepresentation of phylum Firmicutes compared to healthy controls. PSC had a reduced abundance of Prevotella and Roseburia (short chain fatty acid producer) in the mucosa along with an increase in *Enterococcus, Fusobacterium, and Streptococcus* in stool. Intestinal Th17 cells can be induced in mice by segmented filamentous bacteria (SFB), which are spore-forming gram-positive bacteria located in the terminal ileum of the small intestine (80, 84). It is however, unclear if the dysbiosis seen in both PSC-IBD and UC can result in similar changes in mucosal immune profile. Reductions in butyrate producing gut microbial species, as seen in

both PSC-IBD and UC, has been shown to be associated with an increase Th17 cells and induction of release of IL17 from CD4 T cells (231-233). Gut bacteria may not be the sole trigger for these findings. Oxysterols are intermediates in the biosynthesis of bile acids. They have been shown to be natural RORγt agonist ligands and promote differentiation of Th17 cells to drive both innate and adaptive IL-17–dependent immune responses (234, 235). A study recently showed that specific bile acids metabolites control Th17 and Treg differentiation through its direct interaction with RORγt (236). Patients with PSC have changes in faecal bile acid compositions compared to those with UC alone and healthy controls. This is likely to be both a result of cholestasis as well as changes associated with bile salt deconjugation and biotransformation (primary to secondary bile acids) by PSC-IBD specific gut bacteria expressing bile salt hydrolase and hydroxysteroid dehydrogenases (237). These findings may highlight an additional potential mechanism for the immunological findings seen in PSC-IBD.

We found a significant increase in regulatory T cell populations in UC when compared to healthy controls. A similar trend was seen in PSC-IBD however this failed to reach statistical significance. This paradoxical increase has been demonstrated by multiple earlier studies in where Tregs represent a greater fraction of intestinal lamina propria CD4+ T cells in IBD than healthy controls (99, 238). Moreover, IBD patients have been shown to demonstrate an increased prevalence of circulating IL-17 secreting Foxp3 expressing CD4+ T cells (239). Comparable to our data, colonic inflammation has not been shown to change the proportion of Tregs in IBD. If the Tregs have functional deficits that result in a failure to regulate inflammation remains unclear.

We found a significant reduction in Th1 cell populations in both PSC-IBD and UC in comparison to healthy controls and this is likely to be a consequence of an expansion in the

Th17 subset. Although we did not find any difference in expression of IFNy and TNFa, there was a very significant increase in IL17/IFN dual producing CD4+ T cells in both PSC-IBD and UC when compared to healthy controls (100, 240). T helper cell plasticity is a potential mechanism leading to enrichment of specific T helper cell subsets under certain conditions. Non-classic Th1 cells have been described in literature as expressing the Th17 master transcription factor RORγt and producing IFNy (241, 242). This unique subset has been shown to demonstrate substantial plasticity towards the Th17 phenotype consequently resulting in production of both IL17 and IFNy as part of its pro-inflammatory response.

Our cell isolation protocol using a combination of enzymatic digestion and mechanical dissociation facilitated us to consistently obtain yields of over 1.5 million cells from just three colon biopsy pairs. Consequently, we were able to identify most immune subsets with fair certainty. Characterisation of Th17, Th1 and Th2 subsets were limited to chemokine expression based on the standardised flow cytometry panels from the Human Immunophenotyping Consortium (243, 244). Apart from Tregs, which were characterised by expression of FoxP3 in CD127-CD25+ CD4 cells, we were not able to obtain convincing expression of any other transcription factors including RORγt. Additionally, due to the detection limits with the number of fluorochromes in flow cytometry, the intracellular cytokines were gated on CD4 T cells. Although IL17 is a Th17 signature cytokine, it is unclear how much of the increase in IL17 expression in both PSC-IBD and UC is directly associated with an increase in Th17 cell population. Future studies should consider using CyTOF for higher resolution and detailed analysis of CD4 subset expression in these cohorts (245).

4.5 Conclusions

In conclusion our study demonstrates for the first time that the colonic mucosal immune response in PSC-IBD is characterised by significantly higher Th17 cells and lower Th1 cells compared to controls. Patients with PSC-IBD have higher IL-17 and IL17/IFNγ dual producing CD4 cells. Similar findings are observed in UC and no significant differences were identified between PSC-IBD and UC. Our findings highlight the need to explore the role of key players such as the gut microbiome, bile acids and microbiota derived metabolites in mucosal T cell homeostasis and Th1/Th17 plasticity in PSC.

CHAPTER 5

Clinical outcomes and host-microbiota response to treatment with faecal microbiota transplantation in patients with active ulcerative colitis

(A sub-study of the STOP-Colitis open-label pilot)

5.1 Abstract

Introduction

Imbalance of the gut microbiome is key to the pathogenesis of ulcerative colitis (UC). Published datasets show a positive signal for the use of FMT to treat UC, but the optimal route and dose of FMT remains unanswered. Furthermore, there is very limited data exploring key mechanisms around the host biological response to FMT.

Methods

A prospective, multi-centre open-label, randomised pilot study (STOP-Colitis) assessed two possible routes of FMT delivery, via the naso-gastric route or by delivery to the colon, in 30 patients with active UC recruited from three sites in the UK. Patients were administered either via 8 naso-gastric infusions given over 4 days and then again for 4 days in week 4 for foregut delivery (total of 240g of stool) or via one colonoscopic infusion followed by 7 weekly enemas according to the hindgut protocol (total of 360g of stool). Patients were followed up weekly for 8 weeks.

This chapter is a sub-study of the 17 patients recruited at the Birmingham site and in addition to clinical outcomes, exploring changes in colonic mucosal and peripheral blood mononuclear CD4 subset and regulatory T cell subset populations and colonic mucosal transcriptomic profiles.

Results

17 patients were recruited into the study with 8 randomised to the colonic arm and 9 into the nasogastric arm. One patient terminated early in the colonic arm and 4 patients terminated early in the nasogastric arm. On intention to treat analysis seven (41.1%) entered clinical remission – 5/8 (62.5%) were in the colonic route and 2/9 (22.2%) in the nasogastric arm of the study. Clinical response was achieved in eight (47%) of which 6/8 (75%) were in the colonic route and 2/9 (22.2%) in the nasogastric arm of the study. The differences between route of delivery and clinical outcome were not considered to be significant. Analysis of colonic mucosal lamina propria mononuclear cell populations revealed a significant increase in regulatory T cell populations, effector memory regulatory T cell populations, gut homing regulatory T cell populations and IL-10 producing CD4 T cells in responders. Furthermore, there was a significant reduction in colonic mucosal Th17 cell population, IL-17 producing CD4 T cells and CD8 cell populations. Colonic mucosal transcriptomics revealed that responders to FMT at week 8 had significant downregulation of antimicrobial defence and proinflammatory immunological pathways and an increase in butanoate metabolic pathways compared to both baseline and week 8 non-responders.

Conclusion

This study provides the most comprehensive evidence to date on changes in host biology following FMT in patients with ulcerative colitis. Response to FMT is associated with a significant increase in mucosal regulatory T cells population and butanoate metabolism and a reduction in Th17 cell population and anti-microbial defence and proinflammatory immune pathways.

5.2 Introduction

Accumulating data suggests that alteration in the gut microbiome plays a central and likely causative role in the development and progression of UC (246). Evidence from animal studies and limited datasets from clinical studies highlights the importance of species belonging to Clostridia clusters and short chain fatty acids in mediating colonic inflammation in UC; possibly by increasing immunoregulatory mechanisms (137, 247, 248). Attempts to alter the microbiome with probiotics, whilst disappointing in CD, have shown some promise in UC (249). Since the first exploratory use of FMT to treat UC in 1989, numerous case series and four randomised controlled trials (RCTs) of FMT for the treatment of UC have been published demonstrating encouraging efficacy signals (127, 250-253). A meta-analysis of these RCTs showed that at 8 weeks, 37% of FMT participants achieved remission compared to 18% in the control arm (254, 255). The pooled rate for achieving the combined clinical and endoscopic remission in these studies is 27.9% for those receiving FMT and 9.5% in the control interventions, with a number needed to treat of 5.

These studies are however very heterogeneous in their design with significant differences including in mode of delivery, number of FMT infusions, definition of primary and secondary outcomes making it challenging to compare studies. Furthermore, there is a paucity of data exploring host biological / immunological changes as a consequence of FMT in clinical studies in IBD. Limited evidence from a few case series and animal model studies collectively suggests that FMT potentially mediates its therapeutic effects in IBD by increasing immunoregulatory mechanisms. The causal link, however, is far from being established and certainly it remains to be determined what key players in the constituents of FMT are responsible to this effect.

As a result of this recent work, there is great interest in FMT as a possible treatment for UC, but the optimal route of delivery remains unknown. The STOP-Colitis trial is a multi-centre study evaluating the efficacy of FMT for the treatment of UC (CI: Professor Tariq Iqbal). The trial is divided into two phases – an initial open label pilot study to determine the optimum route of FMT delivery comparing naso-gastric versus colonoscopic delivery and assess the feasibility (recruitment, protocol adherence) of undertaking a trial of FMT in UC. This will then be used to inform the next phase of the trial – a full-scale double-blind RCT of FMT versus placebo, we need to undertake a pilot study.

As part of my PhD, I co-ordinated delivery of the pilot phase at Birmingham (main site) and my role as a research fellow included FMT donor recruitment and screen, identification and recruitment of UC patients from clinics followed by screening and trial registration, endoscopic examination of colon / delivery of FMT, assessment of response to FMT and monitoring / responding to any adverse events. Specific to my PhD I conducted colonic mucosal and peripheral blood immunophenotyping and colonic mucosal transcriptomics to explore changes in host-microbiota interactions in relation to FMT in patients with UC; specifically focusing on the mucosal immune response.

In this Chapter I present a brief study protocol (specific to my PhD thesis), clinical outcomes of patients that I was involved with at Birmingham, along with the basic science exploratory findings. The full pilot protocol for STOP-Colitis has now been published (*Quraishi MN, Yalchin M, Blackwell C, Segal J, Sharma N et al. STOP-Colitis pilot trial protocol. A prospective, open-label, randomised pilot study to assess two possible routes of Faecal Microbiota Transplant delivery in patients with ulcerative colitis. BMJ Open. 2019 Nov 11;9(11):e030659*) and is accessible via https://bmjopen.bmj.com/content/9/11/e030659

5.3 Brief study protocol

5.3.1 Study design

A prospective, multi-centre open-label, randomised pilot study to assess two possible routes of FMT delivery for the treatment of UC. Thirty patients with active UC were randomised to receive FMT via either the naso-gastric route or by direct delivery to the colon. Patients were recruited from three hospitals in the United Kingdom (Queen Elizabeth Hospital, Birmingham; St Mark's Hospital, London; and Glasgow Royal Infirmary). This Chapter presents clinical and mechanistic data of patients recruited at Birmingham.

5.3.2 Sample size

As this is a pilot study, no formal sample size calculation was undertaken as the study is not designed or powered to detect a statistically significant difference in efficacy between the two FMT methods of delivery. The recruitment target for this study is 30 patients.

5.3.3 Aims, objectives and outcome measures

The aims of this pilot study were:

i) to explore clinical outcomes and differences associated with delivery of FMT via nasogastric or colonic routes

ii) to explore changes in host mucosal biology in response to treatment with FMT

In order to achieve these aims, the pilot study had the following clinical (quantitative) and mechanistic objectives.

Clinical objectives

To assess:

- Whether FMT by the NG route induces clinical response in patients with active UC;
- Whether FMT by the colonic route induces clinical response in patients with active UC;
- Tolerability and safety

Mechanistic objectives

To assess:

- Whether FMT by either route is associated with a change in mucosal and peripheral CD4 T cell subsets
- Changes in the colonic mucosal microbiota induced by FMT via each route;
- Changes in the colonic mucosal transcriptome associated with FMT.

Clinical outcome measures

- Clinical response (primary measure of efficacy) defined as ≥3 point reduction in the full Mayo score from randomisation to week 8, and 30% reduction from randomisation and at least 1 point reduction of rectal bleeding sub-score or an absolute rectal bleeding sub-score of 0 or 1;

- Time to clinical response (where clinical response is defined as ≥2 point reduction in partial Mayo);
- Clinical remission at week 8 (full Mayo score of ≤2, with no sub-score >1);
- Adherence to FMT;
- Adverse events (AE) and serious adverse events (SAE).

5.3.4 Donor sample acquisition and processing

Donors were recruited following advertisement from healthy un-related anonymous individuals living in Birmingham. Donor screening was based on rigorous screening using a health-screening questionnaire followed by blood and faecal screen to test for transmissible pathogens in accordance with our recently published UK guidelines and AGA and European guidelines. Individuals who pass the screening process were invited to donate morning faecal samples for 10 days and to deliver these for processing within 6 hours of defaecation.

Donations were processed and stored under an MHRA manufacturing licence at the University of Birmingham Microbiome Treatment Centre. Production of FMT from stool was performed under aerobic conditions and prepared in saline with 10% glycerol added as a cytoprotectant. FMT was then be stored frozen at -80oC for up to 24 weeks. Detailed protocol for preparation of FMT is mentioned in the introduction section of the thesis.

5.3.5 Participants' inclusion / exclusion criteria

Inclusion criteria

- Adult patients (aged between 16 and 70 years) with clinically confirmed UC for at least 12 weeks prior to the screening visit;
- Partial Mayo score of ≥4 and ≤8 despite stable disease maintenance treatment with 5-aminosalicylates with or without immunomodulators, or on no treatment;
- Rectal bleeding subscore of ≥1 on the partial Mayo;
- Able to give written, signed informed consent.

Exclusion criteria

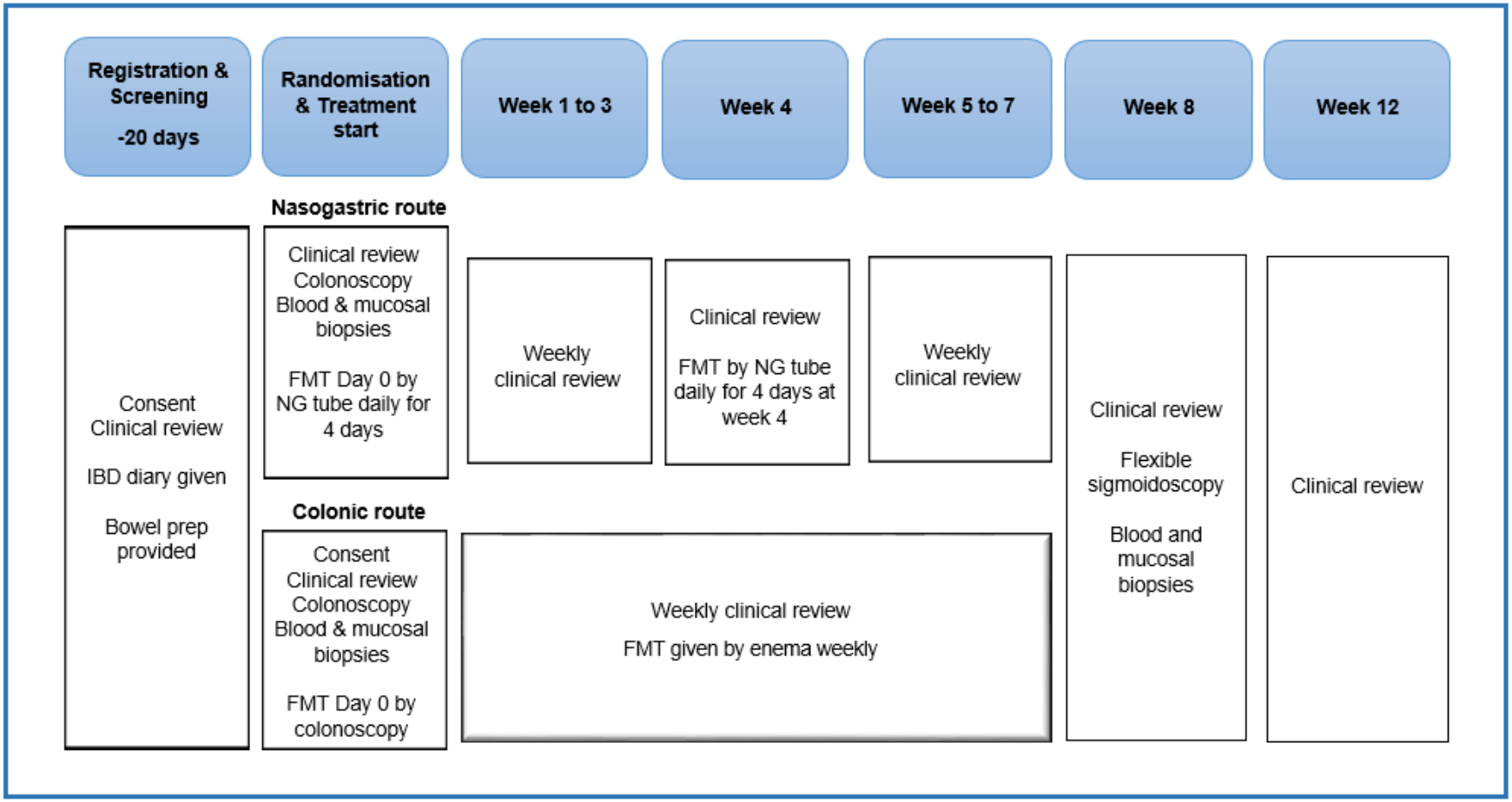
- Stool positive for *Clostridium difficile* or infection by either PCR or ELISA;
- Positive for Hepatitis A/B/C, and/or Human Immunodeficiency Virus (HIV) infection;
- Antibiotics in the preceding 12 weeks prior to date of the screening visit;
- Systemic/topical steroids in the preceding 2 weeks prior to the date of the screening visit;
- Biologics in the preceding 12 weeks prior to the date of screening visit;
- Commercial probiotics and prebiotics in the preceding 12 weeks prior to the date of the screening visit;
- On oral nutritional supplements or enteral/parenteral nutrition in the preceding 4 weeks prior to the date of the screening visit;
- Pregnant or lactating;
- Not willing to take appropriate contraceptive measures to prevent pregnancy during trial participation.

5.3.6 Trial schema

A summary of the trial schema is outlined in Figure 5 - 1.

Figure 5 - 1 : Trial schema and data collection outline

FMT samples will be administered to patients either via eight NG given initially over 4 days starting on the day of randomisation, and then again for 4 days in week 4 for foregut delivery (total of 240 g of stool) or via one colonoscopic infusion followed by seven weekly enemas according to the hindgut protocol (total of 360 g of stool). Patients will be followed up weekly for 8 weeks, and then at 12 weeks. The primary outcome will be a composite assessment of both qualitative and quantitative data based on efficacy (clinical response), acceptability and safety.



5.3.7 Participant enrolment

Potentially eligible patients who express an interest in participating in the trial were consented via a two-stage consent process. Participant information sheets were provided to facilitate the consent process. The first stage (*registration and screening visit)* involved consent for trial specific screening activities as well as basic physiological assessments and baseline blood tests. A diary to record bowel symptoms in order to assess the partial Mayo score was provided. Following this screening visit, if eligibility was confirmed that patient took part in the second stage of consent for entry into the trial and randomization (nasogastric versus colonic). Randomisation was performed by a computer-generated program at the Birmingham Clinical Trials Unit (BCTU).

5.3.8 Interventions

Before the initial FMT treatment, all patients received standard bowel preparation: 2 litres of reconstituted Moviprep® solution within the 24 hours prior to procedure. All patients underwent a colonoscopy at baseline to assess disease, so that a full Mayo score can be calculated, and to collect mucosal biopsies for mechanistics.

<u>Naso-gastric treatment</u>

Patients randomised to nasogastric treatment route were pre-treated with a proton pump inhibitor (lansoprazole 30mg) and a prokinetic agent (domperidone 10mg) at least 30 minutes prior to each FMT infusion to reduce gastric acid secretion and prevent the risk of gastro-oesophageal regurgitation. Following thawing at room temperature, 50ml thawed FMT containing 30g faeces was infused. Following the first treatment, patients returned for the next

three days for further treatment following an overnight fast. At week 4, patients will return for a further 4 FMT infusions over 4 consecutive days following a fast from midnight.

In summary, patients received 30g FMT in 50ml aliquots for NG administration each day for 4 days at the start of the trial (starting on the day of randomisation), and then again for 4 days in week 4 (total FMT dose 240g).

Colonoscopic treatment

Patients randomised to the colonic treatment received a thawed 250ml aliquot of FMT (containing 150g faeces) on the day of randomisation. This was delivered to the colon using a spray catheter, with 125ml sprayed into the caecum to treat the right side of the bowel and the remaining 125ml sprayed directly onto the rest of the colon. Following treatment, patients received a single dose of loperamide. Patients then returned on a weekly basis to receive 100ml enema up to week 7 containing 50ml of saline and 30g faeces, followed by loperamide each time. A single dose of loperamide in order to facilitate prolonged retention of the product has been given in most of the colonic delivered FMT trials in UC and has not been shown to be associated with adverse outcomes.

In summary, patients received 150g of stool in 250ml for colonic administration on the day of randomisation followed by 30g of stool in 100ml in normal saline for administration by enema for 7 weeks (total FMT dose 360g).

5.3.9 Data collection

All patients were followed weekly up to week 8, and then again at week 12. At each visit, the partial Mayo score was calculated from the patient diaries, and medication use and any adverse events occurring recorded. At week 8, a flexible sigmoidoscopy was undertaken to calculate the full Mayo Score and take colonic mucosal biopsies for mechanistic analysis.

5.3.10 Exploratory mechanistic analysis (detailed methodology as per Chapter 2)

Changes in colonic mucosal and peripheral CD4 immune cells subset

Colonic lamina propria mononuclear cells (from sigmoid biopsies) and peripheral blood mononuclear cells at baseline and week 8 will be investigated to identify changes in CD4 cell subsets, regulatory T cell subsets and specific cytokine producing CD4 cells.

Changes in colonic mucosal transcriptomic response

3' RNA-sequencing will be performed on biopsies taken from sigmoid at baseline and at week 8 in order to identify differentially expressed genes and pathways associated with FMT.

Changes in colonic mucosal microbiota profiles

16S ribosomal DNA sequencing will be performed on mucosal biopsies taken from the right colon, left colon and rectum at baseline and from left colon and rectum at week 8 in all patients. Together these samples will be compared to clinical data, and correlations found in order to find the samples most likely to be biologically informative as a marker of disease remission.

5.3.11 Ethics and dissemination

The trial was performed in accordance with the recommendations guiding physicians in biomedical research involving human subjects, adopted by the 18th World Medical Association General Assembly, Helsinki, Finland, June 1964, amended at the 48th World Medical Association General Assembly, Somerset West, Republic of South Africa, October 1996.

The trial was conducted in accordance with the Research Governance Framework for Health and Social Care, the applicable UK Statutory Instruments (which include the Medicines for Human Use Clinical Trials 2004 and subsequent amendments and the General Data Protection Regulation (GDPR) (EU) 2016/679 and Human Tissue Act 2008) and Guidelines for Good Clinical Practice (GCP). This trial was carried out under a Clinical Trial Authorisation in accordance with the Medicines for Human Use Clinical Trials regulations. Trial registration number ISRCTN74072945

Ethical approved for this study has been obtained from the East Midlands-Nottingham Research Ethics Committee (REC 17/EM/0274). Ethics for immunophenotyping and colonic mucosal transcriptomics are as described in methodology.

5.3.12 Funding Statement

This clinical trial is funded by the National Institute for Health Research (NIHR) as part of the Efficacy and Mechanism Evaluation (EME) programme. The immunophenotyping and colonic mucosal transcriptomics were done using local NHS R&D funding.

5.4 Clinical results

5.4.1 Patient characteristics

19 patients were screened and registered at the Birmingham site. Of these 2 failed screening with one patient having been diagnosed with a concurrent enteric infection and another patient having had a rectal bleeding score of 0 on the week prior to randomisation. Of the 17 patients that were successfully randomised 7 (41.1%) were women. The median age of the study patients was 40.1 years (IQR 13.4 years). Following randomisation, 8 entered the colonic arm of the study and 9 entered the nasogastric arm. Five patients withdrew from the study before completing all 8 treatment sets of which 4 were in the nasogastric arm and 1 in the colonic arm. Patient and disease characteristics is shown in the Table 5 - 1 and flow of the study patients at the Birmingham site is shown in Figure 5 - 2.

5.4.2 Donor and FMT characteristics

The FMT treatment sets given to the patients at Birmingham were acquired from 5 donors (mean age 32, 4 male). Of the 17 patients randomised into the study, 10 patients were allocated treatments set from FMT from Donor 13, 2 patients from Donor 15, 1 patient from Donor 16, 3 from Donor 20 and 1 from Donor 21.

Table 5 - 1 : Baseline characteristics of the study groups

Characteristics	Overall	Colonic	Nasogastric
Age, median (IQR), y	40.1 (13.4)	37.2 (4.4)	49.1 (12.2)
Women, No, %	7 (41.1%)	5 (62.5)	2 (22.2)
Men, No, %	10 (58.8%)	3 (17.6%)	7 (41.2%)
UC distribution			
Pancolonic, No, %	9 (52.9%)	4 (50%)	4 (44.4%)
Left sided, No, %	5 (29.4%)	3 (37.5%)	2 (22.2%)
Proctitis, No, %	3 (17.6%)	1 (12.5%)	2 (22.2%)
Medications			
Aminosalicylates	16 (94.1%)	8 (100%)	8 (88.9%)
Thiopurines	9 (52.9%)	4 (50%)	5 (55.6%)
Biologic naïve	9 (52.9%)	4 (50%)	5 (55.6%)
Previous anti-TNF	8 (47.1%)	4 (50%)	4 (44.4%)
Previous vedolizumab	3 (17.6%)	2 (25%)	1 (11.1%)
Baseline characteristics			
Total Mayo at baseline, mean (SD)	7.4 (1)	8 (0.5)	7.5 (1.1)
Stool frequency subscore, mean (SD)	2.1 (0.3)	2 (0)	2.2 (0.4)
Rectal bleeding subscore, mean (SD)	1.6 (0.5)	1.6 (0.5)	1.6 (0.5)
Global assessment subscore, mean (SD)	1.9 (0.4)	2.1 (0.4)	1.8 (0.4)
Endoscopic subscore, mean (SD)	2.1 (0.4)	2.3 (0.5)	1.9 (0.3)

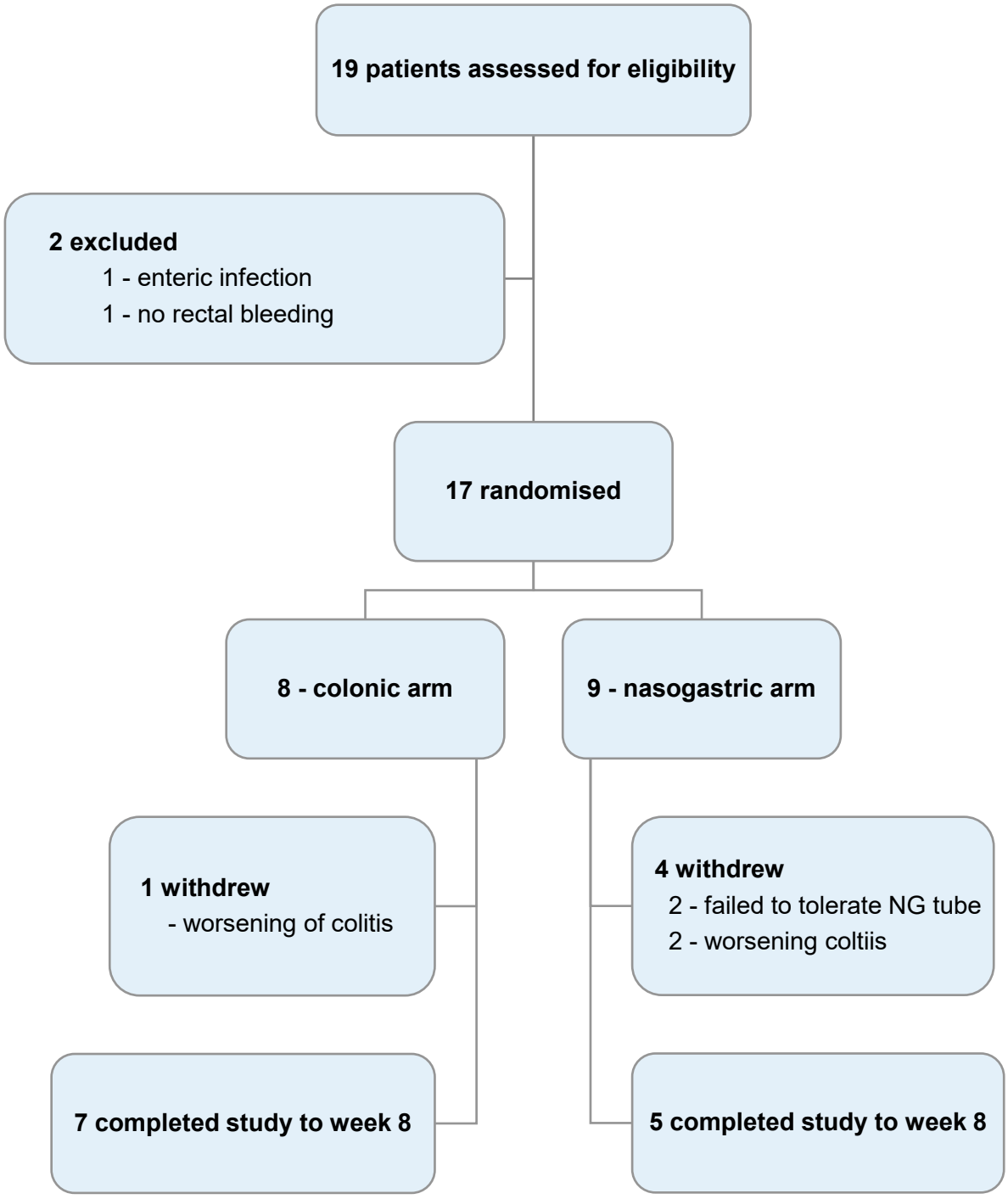


Figure 5 - 2 : Flow of patients in the STOP-Colitis pilot study at the Birmingham site

5.4.3 Clinical remission

Clinical outcomes from the substudy (Birmingham cohort) are shown in Table 5 - 2. On intention to treat analysis, of the 17 patients enrolled in the study seven (41.1%) entered clinical remission – 5/8 (62.5%) were in the colonic route and 2/9 (22.2%) in the nasogastric arm of the study. On analysis per protocol 58.3% (7/12) patients overall of which 71.4% (5/7) of patients in the colonic arm and 40% (2/5) in the nasogastric arm achieved clinical remission. This higher remission rate in the colonic arm was not statistically significant when analysed either by intention to treat or per protocol (p = 0.15 and 0.56 respectively). The overall mean time to sustained clinical remission was 4 weeks (95% CI 5.31 – 2.69). This was not different when analysed by route of delivery – 4.4 weeks (95% CI 5.5 – 3.2) by colonic arm and 3 weeks (95% CI 4.48 – 1.52) by nasogastric arm; P = 0.43.

5.4.4 Clinical response

On intention to treat analysis, of the 17 patients enrolled in the study eight (47%) achieved clinical response – 6/8 (75%) were in the colonic route and 2/9 (22.2%) in the nasogastric arm of the study. The overall mean reduction in partial Mayo Score was 2.76 (95% CI 4.03 - 1.50). However, the reduction in the mean partial Mayo score in the colonic arm was significantly greater than the nasogastric arm - 4.5 (95% CI 6.27 – 2.73) and 1.22 (95% CI 2.28 – 0.16) respectively; P = 0.009. On analysis per protocol 8/12 (66.7%) of patient over all of which 85.7% (6/7) in the colonic arm and 40% (2/5) in the nasogastric arm achieved clinical response. The difference in clinical response based on route of delivery was almost statistically significant with a p value of 0.056 when analysed by intention to treat and not statistically significant when analysed per protocol (p=0.10). The overall mean time to clinical response was 1.63 weeks (95% CI 2.11 – 1.14). This was not different when analysed by route of delivery - 1.67 weeks (95% CI 1.15 - 2.18) by colonic arm and 1.5 week (95% CI 1.84 -

1.15) by nasogastric arm; P = 0.81. A graphical representation of weekly change in partial Mayo score is demonstrated in Figure 5 - 3 for the colonic arm and Figure 5 - 4 for the nasogastric arm.

5.4.5 Endoscopic remission

On intention to treat analysis, of the 17 patients enrolled in the study four (23.5%) achieved mucosal healing (Mayo endoscopic sub score 0). Of these 2/8 (25%) were in the colonic route and 2/9 (22.2%) in the nasogastric arm of the study. On analysis per protocol 28.5% (2/7) of patients in the colonic arm and 40% (2/5) in the nasogastric arm achieved clinical response. The difference in mucosal healing based on route of delivery was not statistically significant analysed either by intention to treat or per protocol.

5.4.6 Histological remission

Four patients (23.5%) overall achieved deep remission (no histological evidence of inflammation). Of these 2/8 (25%) were in the colonic route and 2/9 (22.2%) in the nasogastric arm of the study. On analysis per protocol 28.5% (2/7) of patients in the colonic arm and 40% (2/5) in the nasogastric arm achieved clinical response. The difference in mucosal healing based on route of delivery was not statistically significant analysed either by intention to treat or per protocol.

5.4.7 Biologic experienced patients

Three out of 5 patients (37.5%; 2 in colonic arm and 1 in nasogastric arm) who were previously exposed and failed biological therapy for ulcerative achieved clinical response and remission failed to respond.

Table 5 - 2 : Clinical outcomes based on intention to treat analysis

	Colonic route	NG route	Overall
Clinical Response	6/8 (75%)	2/9 (22.2%)	8/17 (47%)
Clinical Remission	5/8 (62.5%)	2/9 (22.2%)	7/17 (41.1%)
Endoscopic Remission	2/8 (25%)	2/9 (22.2%)	4/17 (23.5%)
Histological Remission	2/8 (25%)	2/9 (22.2%)	4/17 (23.5%)

5.4.8 Patient acceptability

Of the 5 patients that terminated early from the study 4 had been randomised into the nasogastric arm. 2 of these 4 patients had terminated due to lack of tolerability to the nasogastric tube. One patient from the colonic arm and two from nasogastric arm terminated early from the study due to worsening of colitis. There were no terminations from the study directly attributed to lack of tolerability to FMT.

5.4.9 Management of early terminations and FMT failures

Of the 5 patients that terminated early, 3 had significant worsening of colitis that required rescue oral steroid therapy. The other two patients were commenced on immunomodulator therapy. Of the patients that completed the study, eight patients failed to respond to FMT. Five of these required oral steroids at the end of the study and two were commenced on biological therapy and one on immunomodulator.

5.4.10 Adverse events

Three patients experienced worsening of their colitis that required rescue steroid therapy. None of these patients required hospital admission. Two patients in the nasogastric route experienced mild transient nausea, and three patients experienced mild abdominal pain and bloating after the baseline colonoscopy of which two had FMT via the colonic route. One patient required a hospital admission that was unrelated to the clinical trial. There were no adverse events directly attributable to FMT.

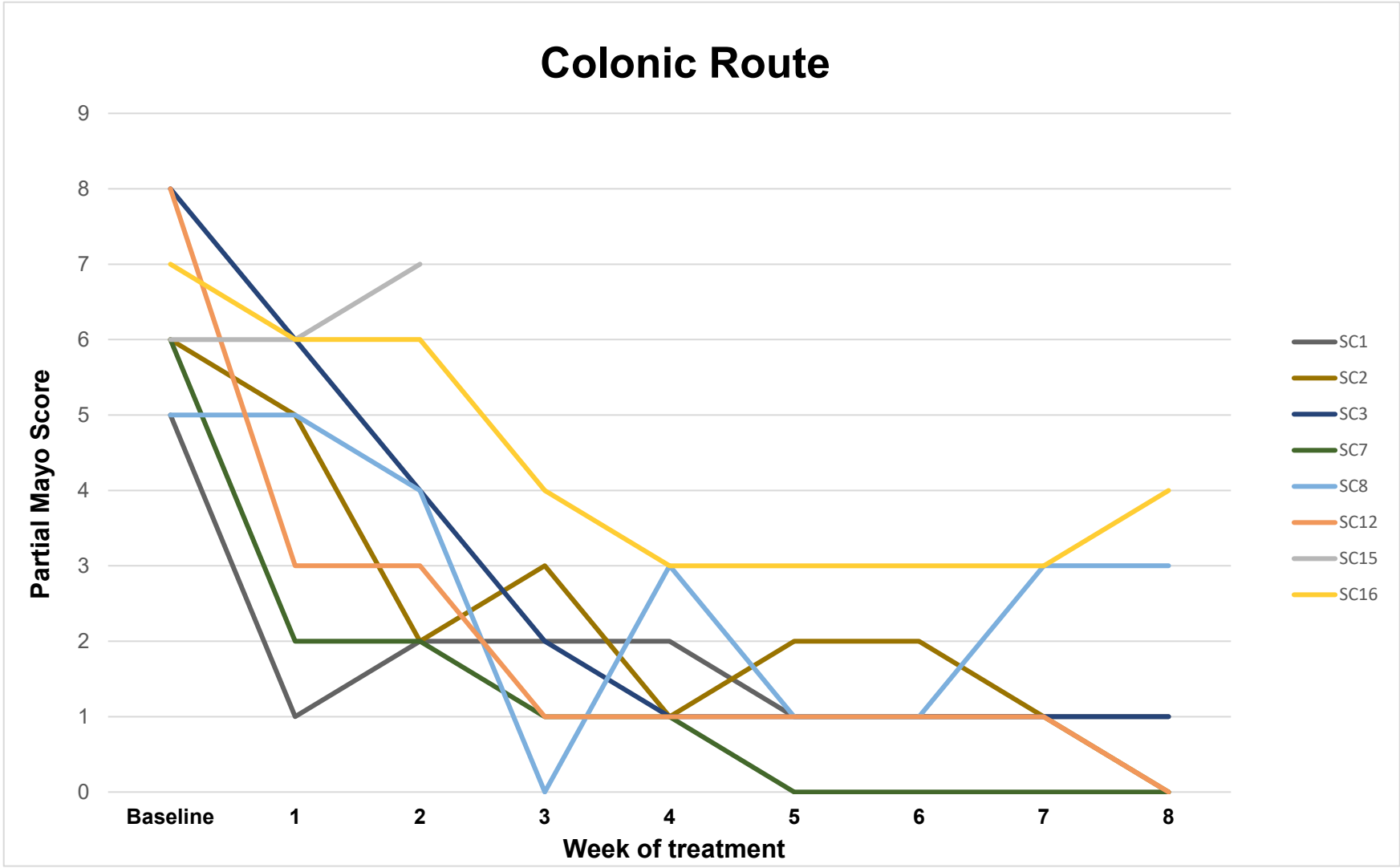


Figure 5 - 3 : Weekly partial Mayo scores of patients randomised to the colonic arm of the study

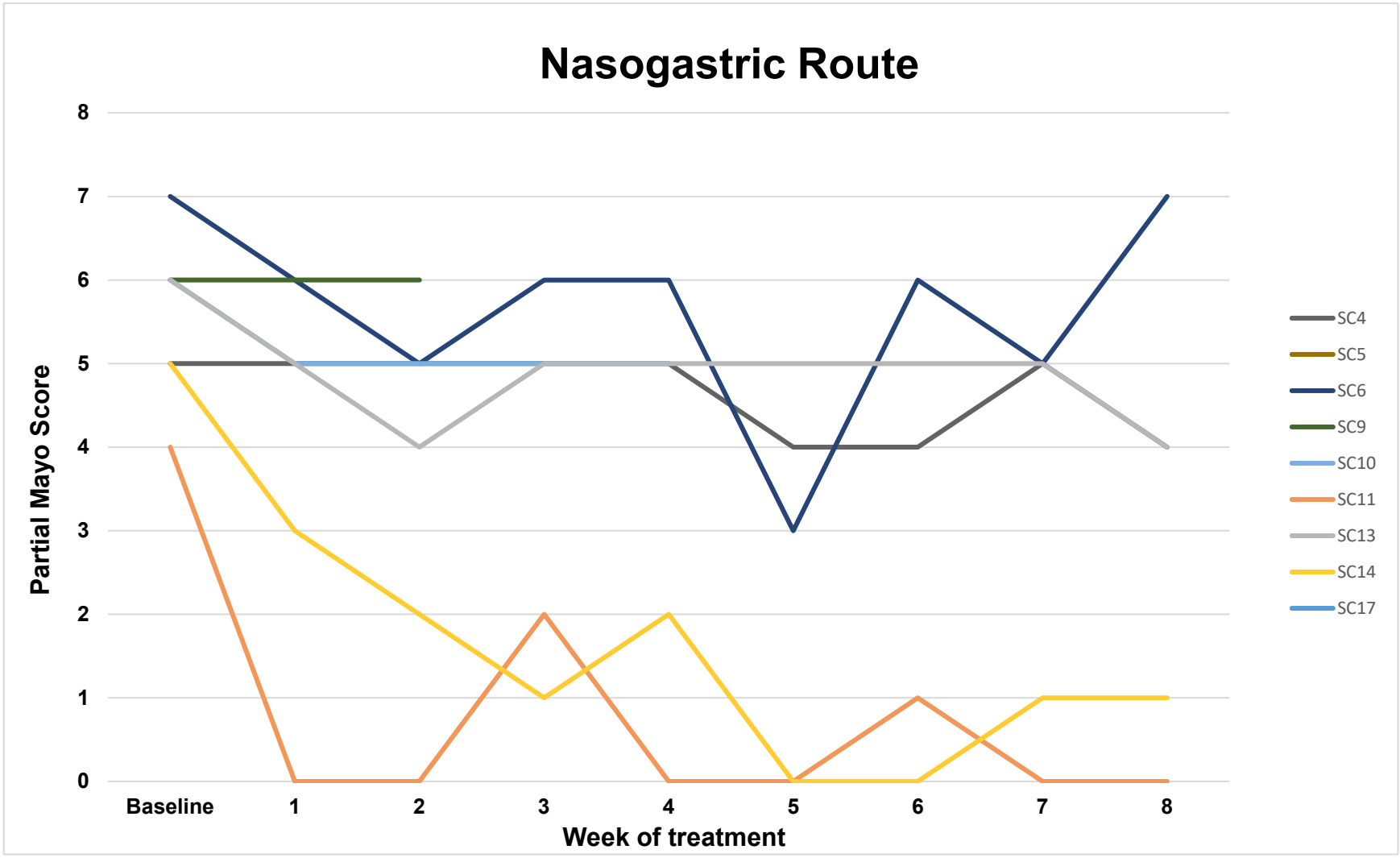


Figure 5 - 4 : Weekly partial Mayo scores of patients randomised to the nasogastric arm of the study

5.5 Changes in colonic mucosal lamina propria mononuclear cell populations associated with treatment with FMT

Based on the animal model studies as outlined in Chapter 1, we had hypothesised that there changes in regulatory T cell population associated with treatment with FMT. As outlined earlier, I used three panels to explore changes in mucosal CD4 T cell subsets, specific cytokine producing CD4 cells and regulatory T cell subset and function panel.

Of the 17 patients randomised into the study, 12 had completed the study to week 8. Biopsies for LPMC characterisation were only obtained for 11 patients however whole blood for peripheral blood mononuclear cells were obtained for all 12 (data for PBMC presented later in section 5.5.5). Due to the degree of compensation being applied during the process of gating in view of reaching the threshold of number fluorochrome antibody panels used for the flow cytometry, the CD4 gating strategies was not clear. A 'fluorescent minus one' with CD4 was used to define the positive and negative population as shown in methods section.

5.5.1 Change in colonic immunophenotyping colonic immune subsets

Mean changes and significance values based on paired t-test analysis in colonic immune subsets at week 8 compared to baseline change is shown in Table 5 - 3. Key findings are detailed below:

Regulatory T cells increase following FMT with a more significant increase in responders

The frequency of regulatory T cells CD127-CD25+FoxP3+ increased significantly at week 8 compared to baseline (delta 5.02%; p=0.01) – Figure 5 - 5. This increase was considerably greater and significant in responders compared to non-responders (delta 5.29%; p=0.009, vs delta 4.55%; p=0.36).

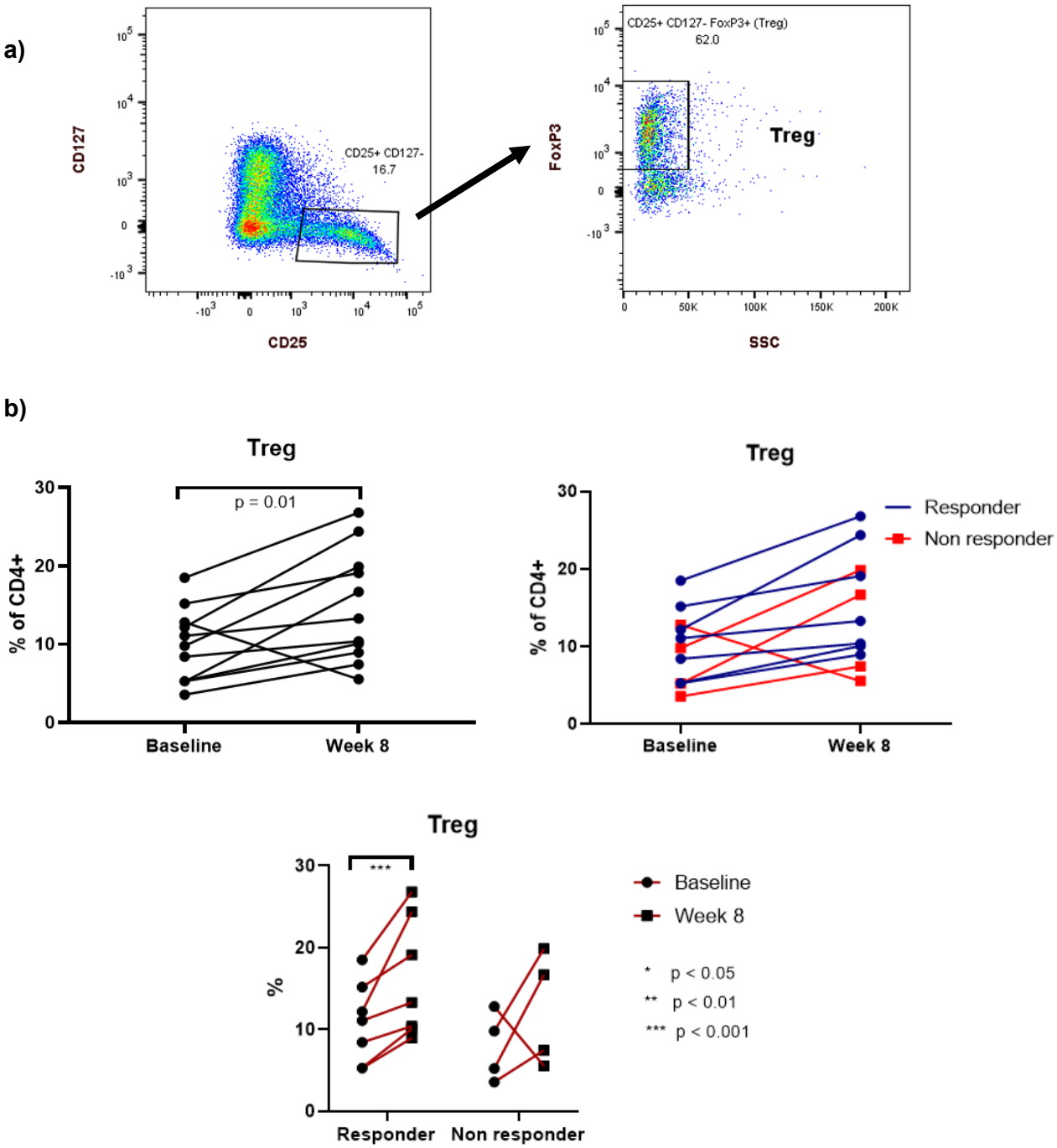


Figure 5 - 5 : Changes in colonic regulatory CD4 T cell frequencies following FMT.

a) Graphical representation of Treg gating. b) Line graph demonstrating change in Treg frequencies in the entire cohort along with subgroup analysis based on clinical response following FMT.

Th17 decrease following FMT with a more significant decrease in responders

The frequency of Th17 cells (CCR+CD161+) decreased significantly at week 8 compared to baseline (delta -5.13%; p=0.03) – Figure 5 - 6. Subgroup analysis revealed that this reduction was significant and greater in responders compared to non-responders (delta -7.61%; p=0.017, vs delta -0.78%; p=0.83).

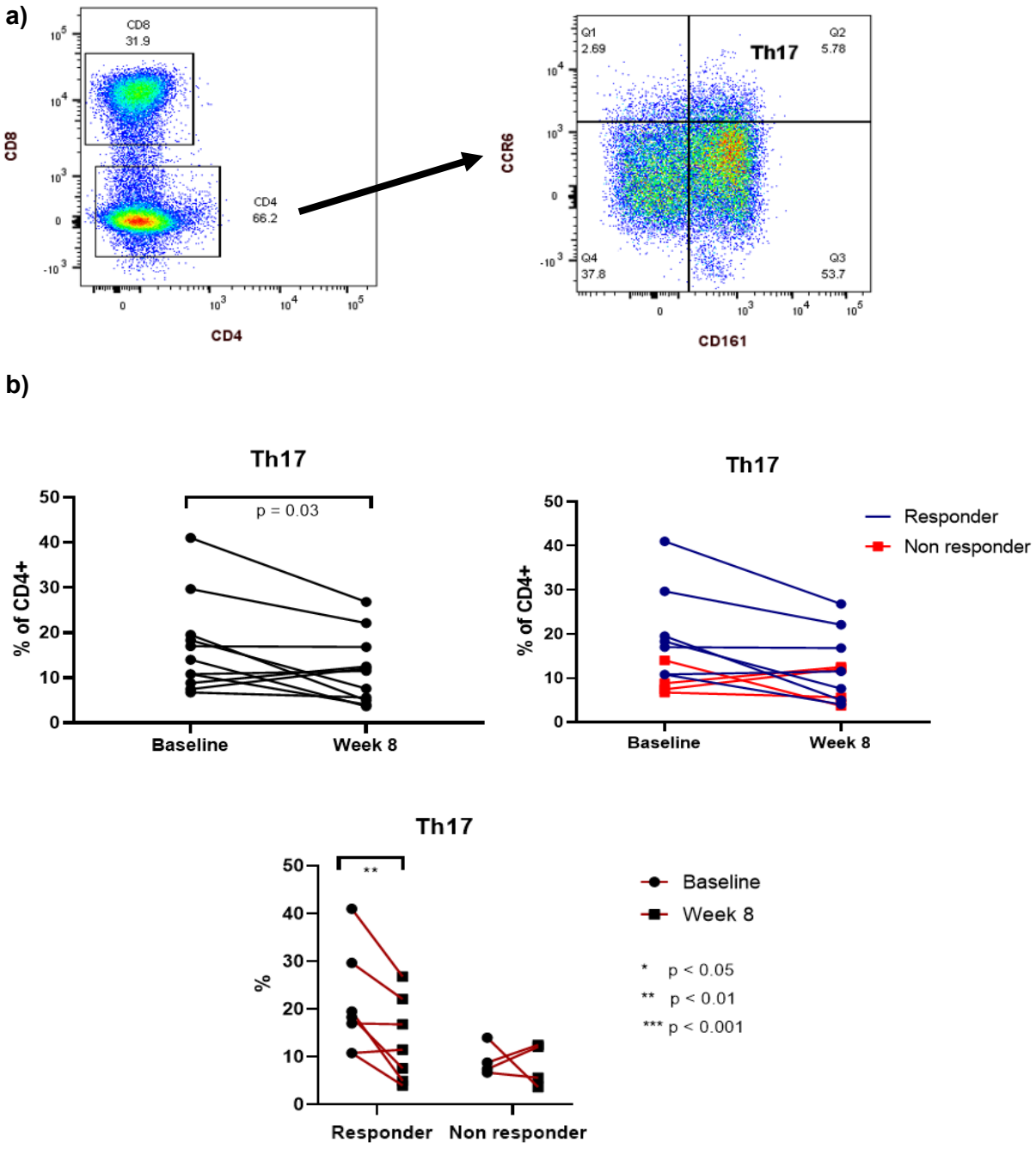


Figure 5 - 6 : Changes in colonic Th17 cell frequencies following FMT.

a) Graphical representation of Th17 gating. b) Line graph demonstrating change in Th17 frequencies in the entire cohort along with subgroup analysis based on clinical response following FMT.

CD8 T cells decrease following FMT with a more significant decrease in responders

The frequency of CD8 T cells decreased significantly at week 8 compared to baseline (delta -4.41%; p=0.01) – Figure 5 - 7. This decrease was again considerably greater and significant in responders compared to non-responders (delta -5.18%; p=0.04, vs delta -3.06%; p=0.22).

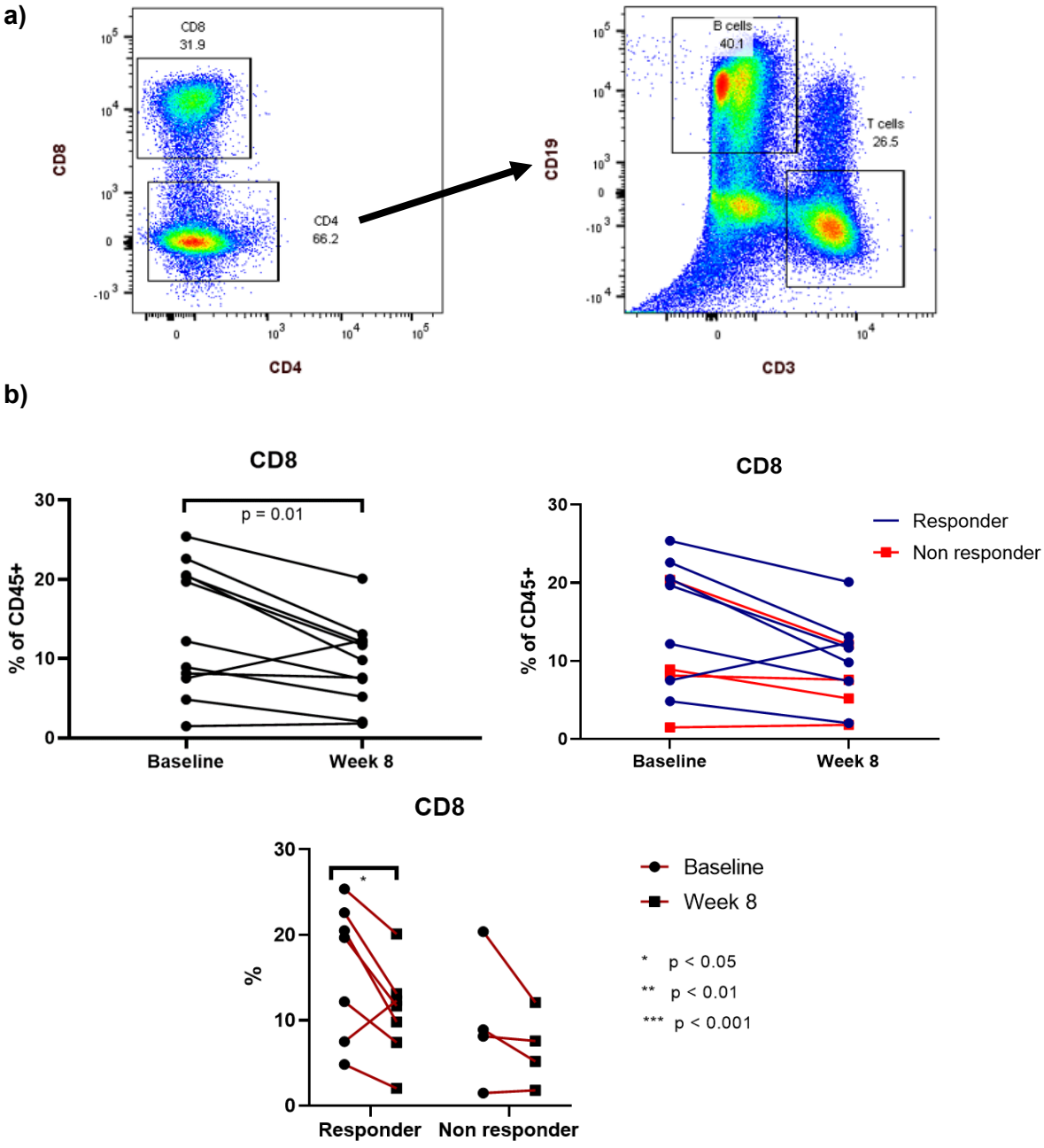


Figure 5 - 7 : Changes in colonic mucosal CD8 cell frequencies following FMT.

a) Graphical representation of CD8 gating. b) Line graph demonstrating change in CD8 frequencies in the entire cohort along with subgroup analysis based on clinical response following FMT.

IL-10 producing CD4 T cells significantly increase in responders following FMT

Consistent with an increase in regulatory T cells, the frequency of IL-10 producing CD4 T cells increase in responders at week 8 compared to baseline (delta 2.16%; p=0.04) – Figure 5 - 8. In contrast there was a reduction in IL-10 producing CD4 T cells in those that failed to respond to FMT, however this was not statistically significant (delta -1.71%; p=0.16).

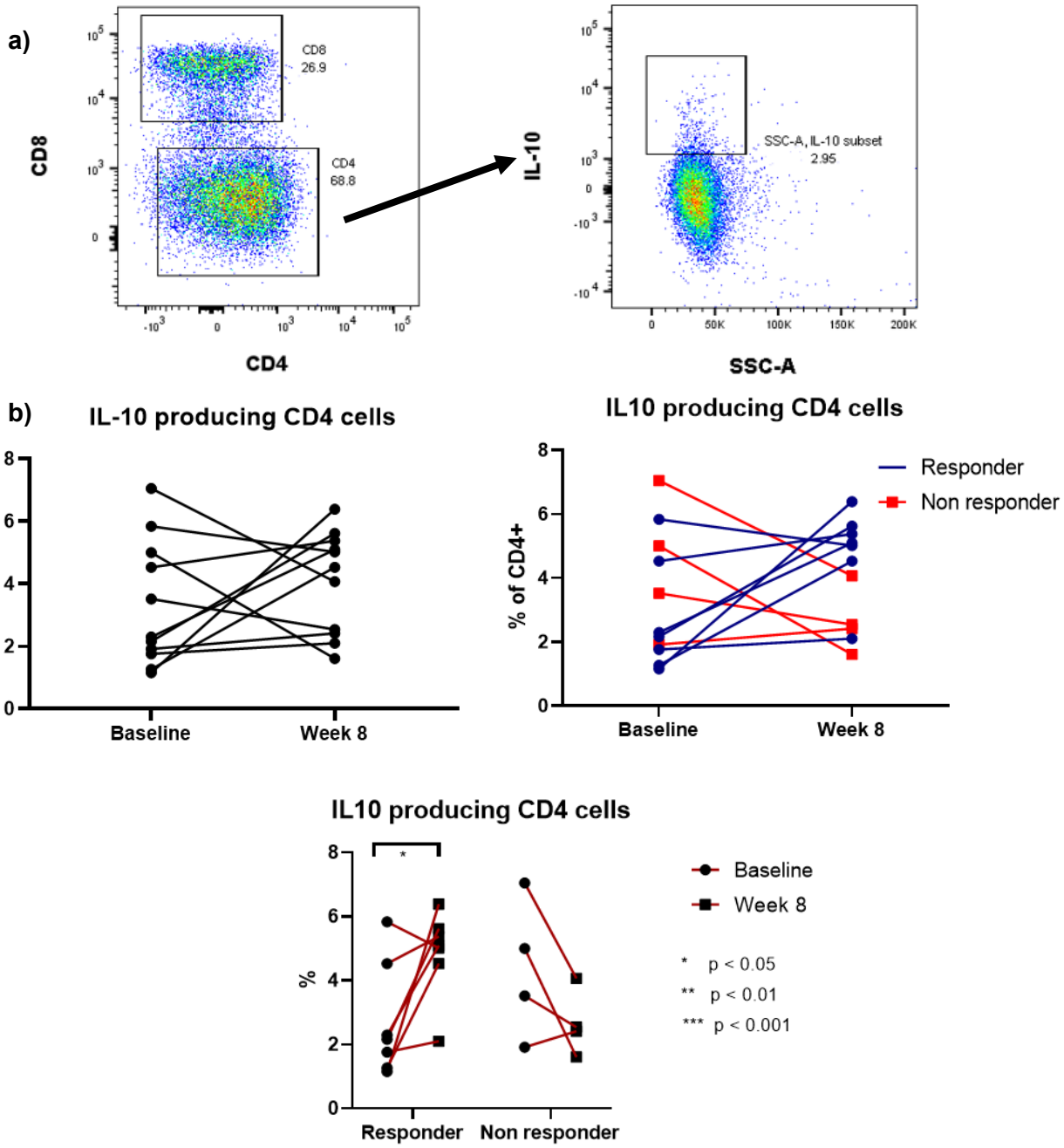


Figure 5 - 8 : Changes in colonic IL-10 producing CD4 frequencies following FMT.

a) Graphical representation of IL-10 producing CD4 cell gating. b) Line graph demonstrating change in IL-10 producing CD4 cell frequencies in the entire cohort along with subgroup analysis based on clinical response following FMT.

IL-17 producing CD4 T cells decrease following FMT with a more significant decrease in responders

The frequency of IL-17 producing CD4 T cells decreased significantly at week 8 compared to baseline (delta -6.41%; p=0.01) consistent with the reduction seen in Th17 cells as shown in Figure 5 - 9. Subgroup analysis revealed that this reduction was significant and greater in responders compared to non-responders (delta -7.69%; p=0.05, vs delta -4.17%; p=0.07).

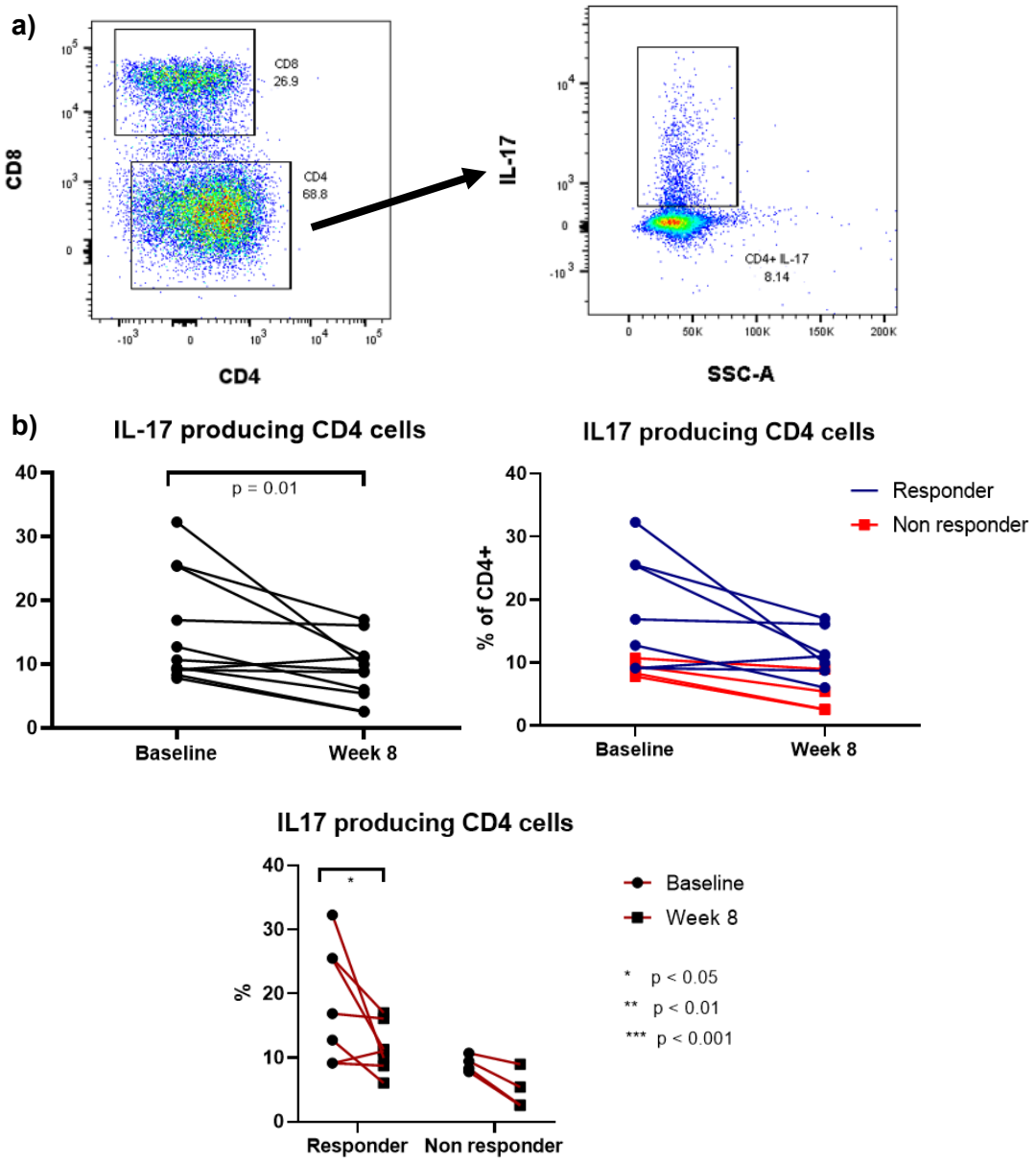


Figure 5 - 9 : Changes in colonic IL-17 producing CD4 frequencies following FMT..

a) Graphical representation of IL-17 producing CD4 cell gating. b) Line graph demonstrating change in IL-17 producing CD4 cell frequencies in the entire cohort along with subgroup analysis based on clinical response following FMT.

Other immune subsets

There was no significant difference seen in frequencies of Th1 like CD4 cells (CCR6-CD161-CXCR3+CCR5+), Th2 like CD4 cells (CCR6-CD161-CXCR3-CCR5-), B cells (CD45+CD19+) at week 8 compared to baseline following treatment with FMT as shown in Figure 5 - 10. Similarly, no difference was seen in frequencies of IFNy, TNFa, IL-5, IL-13 and dual producing IFNy/IL-17 producing CD4 T cells following FMT.

5.5.2 Investigating changes in colonic mucosal regulatory T cell subsets

Based on the animal model studies as outlined in Chapter 1, we had hypothesised that there would be changes in regulatory T cell population associated with treatment with FMT for UC. Consequently, I included a regulatory T cell subset to explore changes in proportions of colonic mucosal and peripheral blood effector and central memory T regulatory cells. Additionally, I investigated changes in proportions of gut homing T regulatory effector memory cells as a result of FMT. These subsets would be of particular interest as they would help better define which subsets are responsible for the significant increase in regulatory T cell population seen following FMT. Gut homing T regulatory effector memory cells were defined as CCR7-CD45-alpha4+ T regs. Experiments with CCR9+, beta 7 (or combination a4b7) antibodies to better define the gut homing populations were attempted, however the the resolution of gating and differentiation of populations were not satisfactory. Whilst accepting that this is not optimal, due to these limitations I decided to exclude these from the panel.

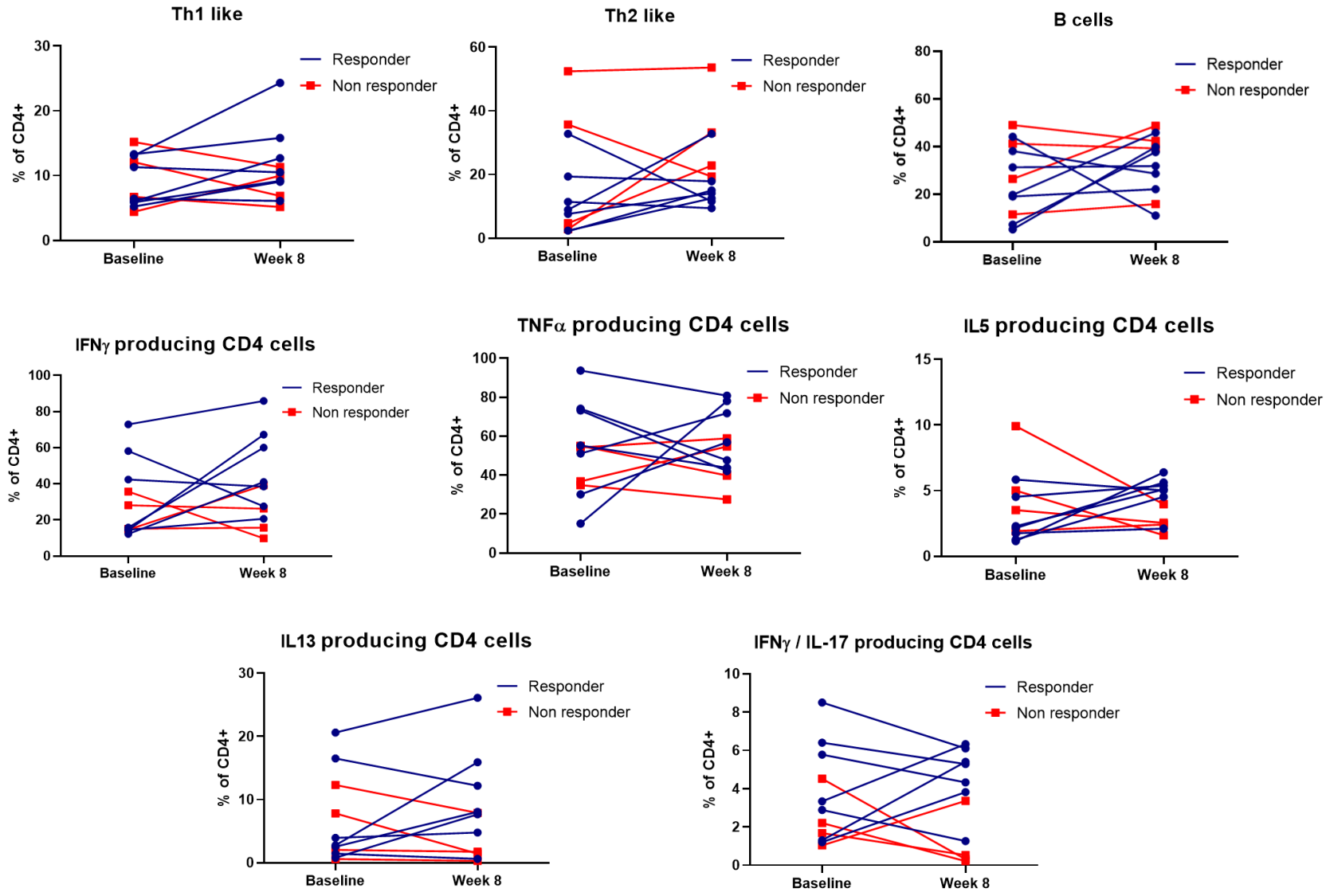


Figure 5 - 10 : Changes in other colonic mucosal immune cell subsets following FMT.

Line graph demonstrating (non-significant) change in other immune cell frequencies based on clinical response following FMT.

Effector memory regulatory T cells increase following FMT with a more significant increase in responders

The frequencies of effector memory regulatory T cells increased significantly at week 8 compared to baseline (delta 7.86%; p=0.01) – Figure 5 - 11. Subgroup analysis based on clinical outcomes revealed that this increase was significant and greater in responders compared to non-responders (delta 12%: p<0.0001, vs delta 0.63%; p=0.92).

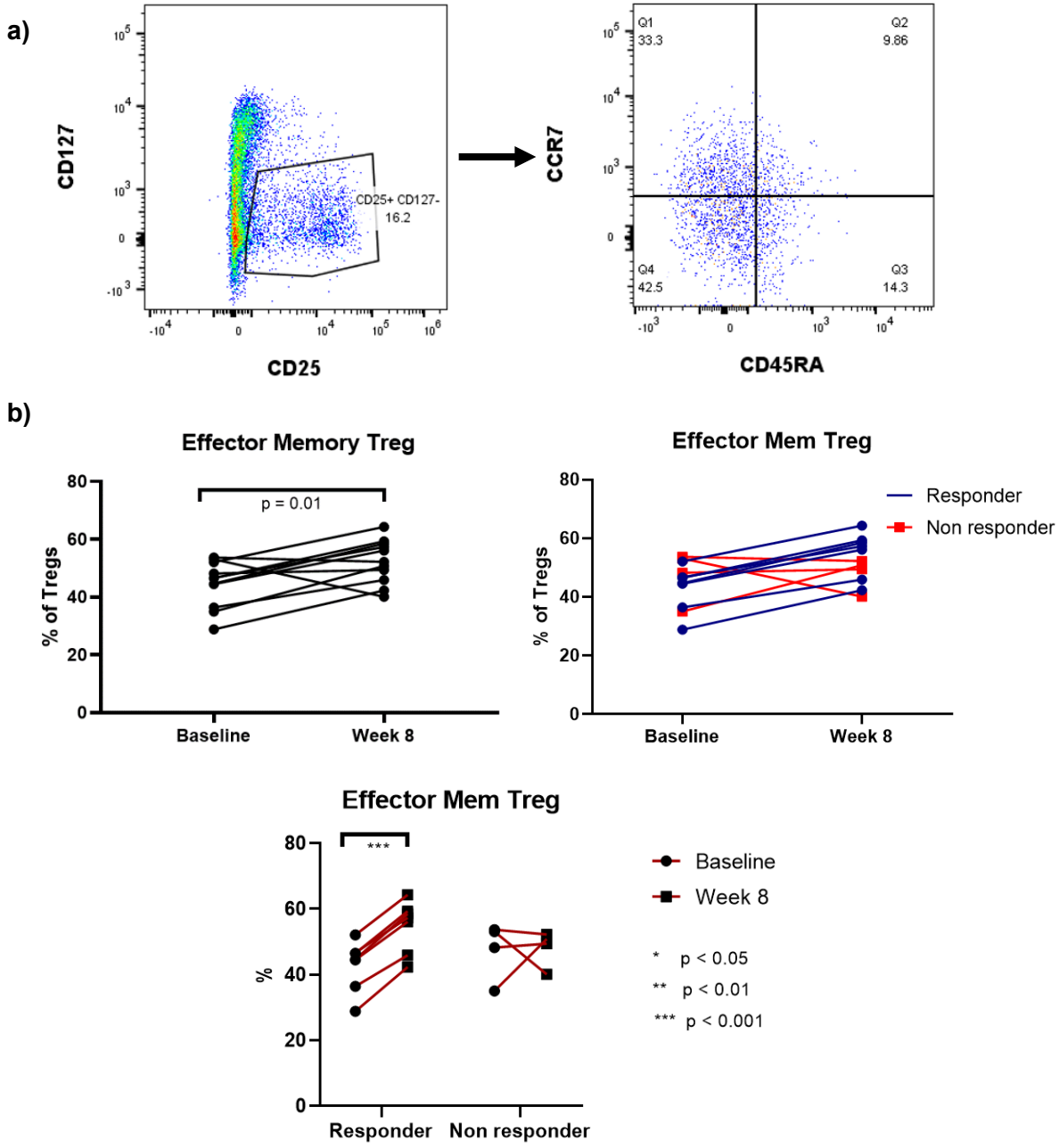


Figure 5 - 11 : Changes in colonic effector memory Treg CD4 frequencies following FMT

a) Graphical representation of effector memory regulatory T cells gating. b) Line graph demonstrating change in effector memory regulatory T cells frequencies in the entire cohort along with subgroup analysis based on clinical response following FMT.

Gut homing effector memory regulatory T cells increase following FMT with a more significant increase in responders

Consistent with the changes seen in the effector memory regulatory T cells population, the frequencies of gut homing effector memory regulatory T cells increased significantly at week 8 compared to baseline (delta 12.12%; p=0.03 – Figure 5 - 12. Subgroup analysis based on clinical outcomes revealed that this increase was significant and greater in responders compared to non-responders (delta 18.55%; p=0.008, vs delta 0.86%; p=0.92).

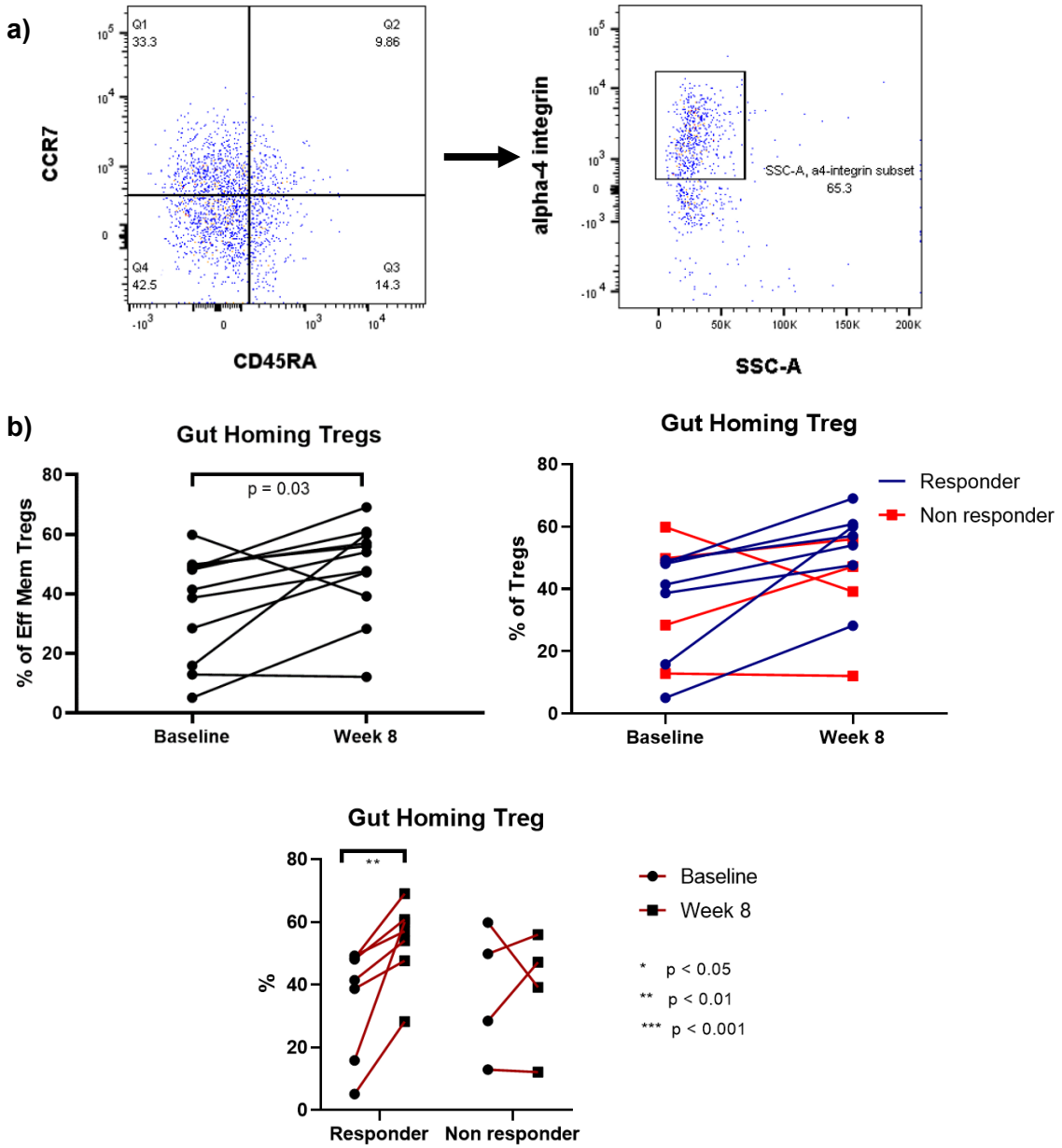


Figure 5 - 12 : Changes in colonic gut homing Treg CD4 frequencies following FMT.

a) Graphical representation of gut homing regulatory T cells gating. b) Line graph demonstrating change in gut homing regulatory T cells frequencies in the entire cohort along with subgroup analysis based on clinical response following FMT.

There is no change in central memory regulatory T cells and naïve regulatory T cells population following FMT

The frequencies of central memory regulatory T cells and naïve Tregs did not change significantly at week 8 compared to baseline (delta -0.39%; p=0.93, delta -2.33%; p=0.22 respectively) regardless of clinical outcome – Figure 5 - 13, 5 - 14.

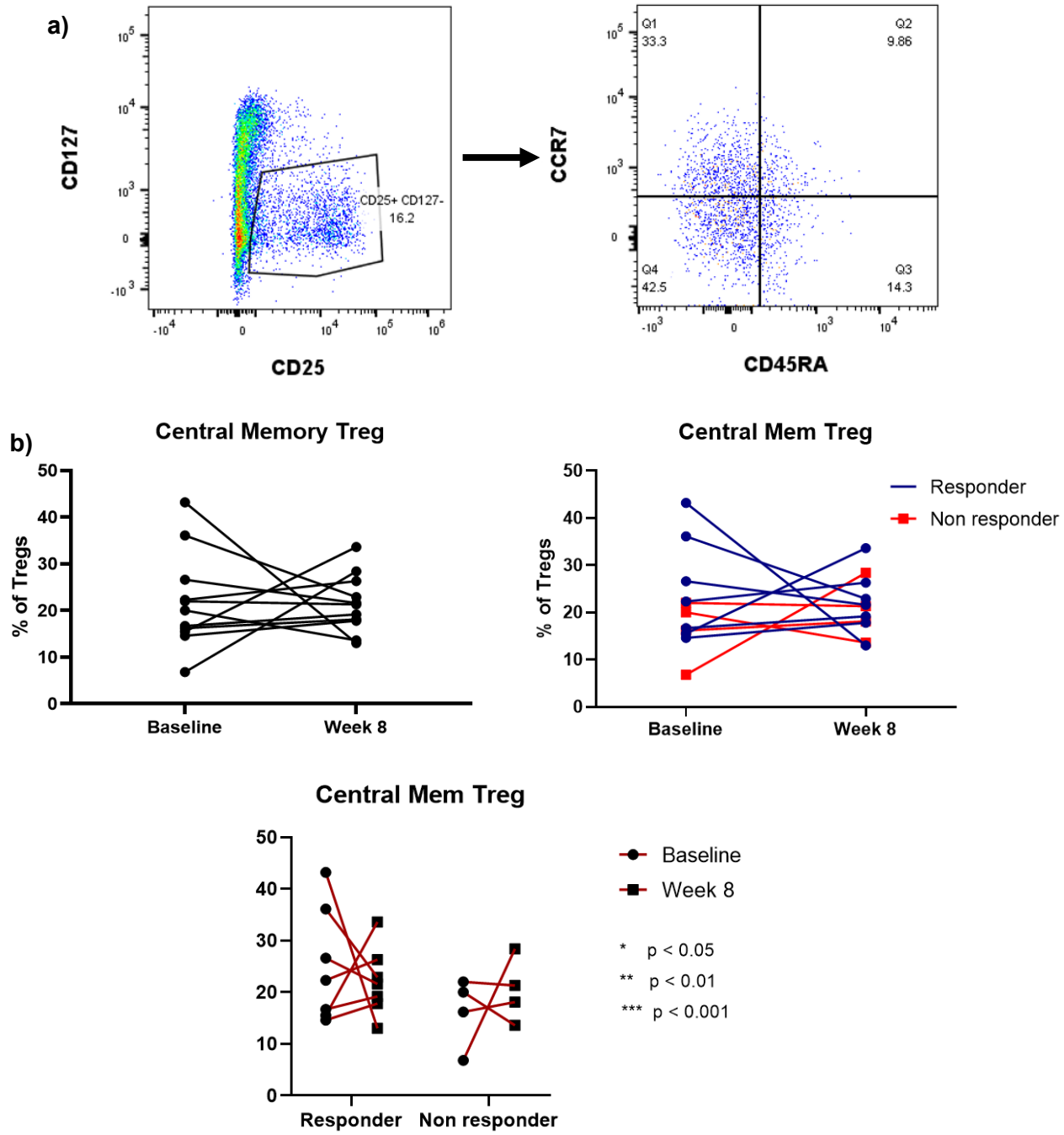


Figure 5 - 13 : Changes in colonic central memory Treg CD4 frequencies following FMT.

a) Graphical representation of central memory regulatory T cells gating. b) Line graph demonstrating change in central memory regulatory T cells frequencies in the entire cohort along with subgroup analysis based on clinical response following FMT.

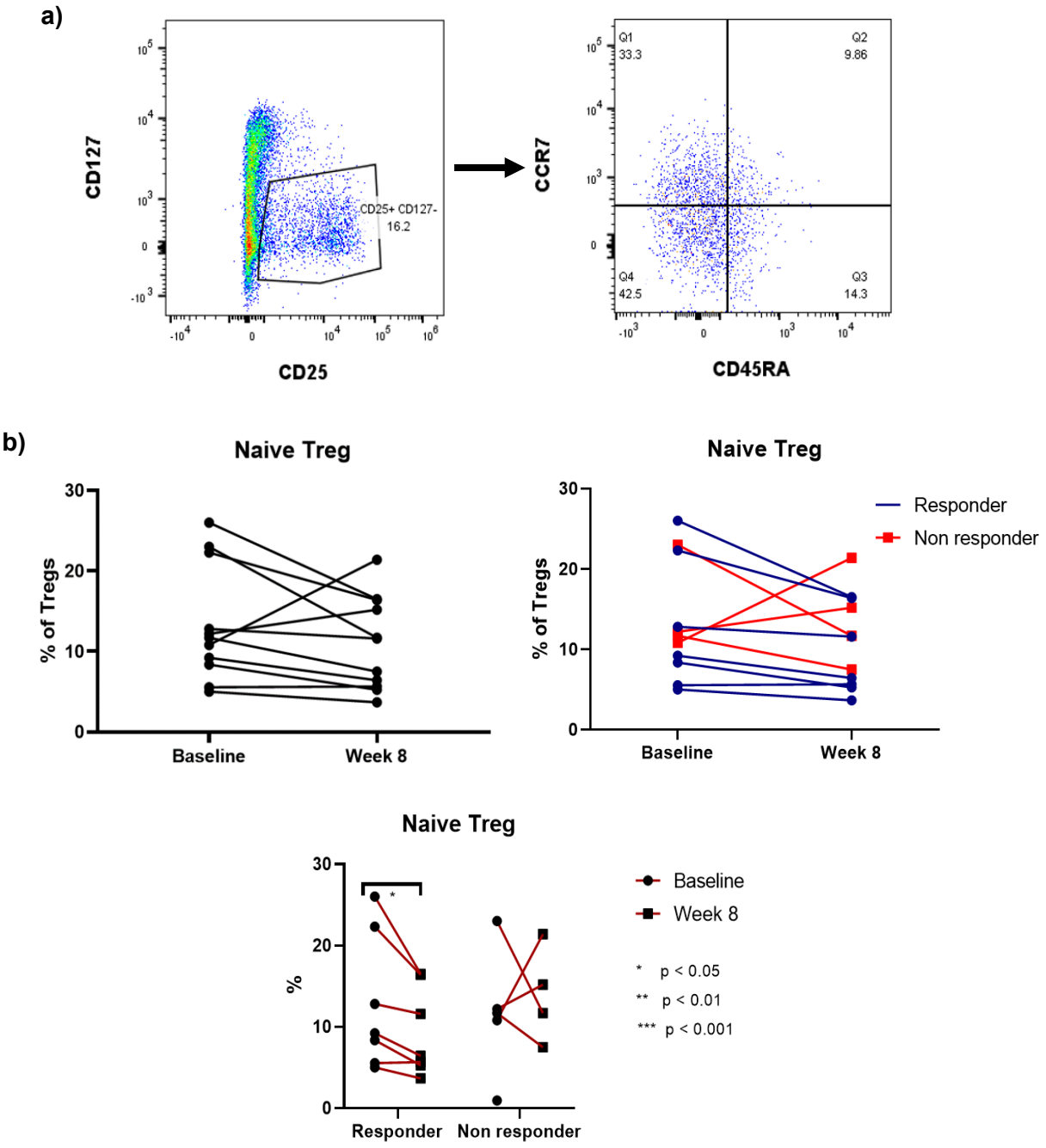


Figure 5 - 14 : Changes in colonic naïve Treg CD4 frequencies following FMT.

a) Graphical representation of naïve regulatory T cells gating. b) Line graph demonstrating change in naïve regulatory T cells frequencies in the entire cohort along with subgroup analysis based on clinical response following FMT.

Table 5 - 3 : Mean change of colonic LPMC subsets at week 8 compared to baseline with subgroup analysis based on clinical response.

	∆ Week 8 compared to baseline in all patients	∆ Week 8 compared to baseline in responders	∆ Week 8 compared to baseline in non-responders
Treg cells	5.02% (P = 0.013)	5.29% (P = 0.009)	4.55% (P = 0.36)
Th17 cells	-5.13% (P = 0.03)	-7.61% (P = 0.017)	-0.78% (P = 0.83)
Th1 cells	1.93% (P = 0.22)	3.77% (P = 0.05)	-1.28% (P = 0.63)
Th2 cells	5.64% (P = 0.26)	4.09% (P = 0.48)	8.35% (P = 0.47)
B cells	6.36% (P = 0.32)	7.44% (P = 0.45)	4.48% (P = 0.53)
CD8 cells	-4.41% (P = 0.01)	-5.18% (P = 0.04)	-3.06% (P = 0.22)
CD8 Tregs	0.58% (P = 0.15)	0.25% (P = 0.59)	1.17% (P = 0.19)
TNF	2.59% (P = 0.76)	4.07% (P = 0.76)	0% (P = 1)
IFN	9.8% (P = 0.25)	15.77% (P = 0.2)	-0.64% (P = 0.95)
IL17	-6.41% (P = 0.01)	-7.69% (P = 0.05)	-4.17% (P = 0.07)
IL10	0.75% (P = 0.38)	2.16% (P = 0.04)	-1.71% (P = 0.16)
IL5	0.11% (P = 0.92)	1.57% (P = 0.16)	-2.45% (P = 0.18)
IL13	1.4% (P = 0.44)	3.82% (P = 0.13)	-2.84% (P = 0.16)
IFN/IL17	-0.17% (P = 0.83)	0.45% (P = 0.67)	-1.26% (P = 0.42)
Naïve Tregs	-2.33% (P = 0.22)	-3.39% (P = 0.03)	-0.48% (P = 0.93)
Central Memory Tregs	-0.39% (P = 0.93)	-2.95% (P = 0.63)	4.09% (P = 0.55)
Effector Memory Tregs	7.86% (P = 0.01)	12% (P = 0.00001)	0.63% (P = 0.92)
Gut Homing Tregs	12.12% (P = 0.03)	18.55% (P = 0.008)	0.86% (P = 0.92)

5.5.3 Investigating functional changes in regulatory T cell subsets

As immunophenotyping data from the first five patients consistently revealed that an increase in regulatory T cell and gut homing effector memory Treg proportions were associated with a clinical response to FMT we decided to add in a panel to explore functional changes in Treg population in the following six patients as shown in Table 5 - 5. These included proliferation marker Ki67, apoptotic markers Bcl-2 and CTLA4 and activation marker CD39.

The frequencies of Ki67 expressing regulatory T cells increased significantly at week 8 compared to baseline (delta 18.97%; $p<0.002$) in clinical responders – Figure 5 - 15. There was no difference seen in non-responders and when analysed as a single cohort.

The frequencies of CTLA4, CD39 and Bcl-2 expressive Tregs did not change significantly at week 8 compared to baseline (delta 4.43%; $p<0.0001$, delta respectively) regardless of clinical outcome.

Table 5 - 5 : Mean change of colonic LPMC Treg functional subsets at week 8 compared to baseline with subgroup analysis based on clinical response.

	∆ Week 8 compared to baseline in all patients	∆ Week 8 compared to baseline in responders	∆ Week 8 compared to baseline in non-responders
Ki67+ Tregs	9.46% (P = 0.175)	18.97% (P = 0.002)	-9.55% (P = 0.34)
Bcl2+ Tregs	-0.82% (P = 0.88)	-8.3% (P = 0.115)	14.15% (P = 0.08)
CTLA4+ Tregs	-5.82% (P = 0.25)	-7.16% (P = 0.33)	-3.15% (P = 0.07)
CD39+ Tregs	1.36% (P = 0.66)	-2.61% (P = 0.36)	9.3% (P = 0.08)

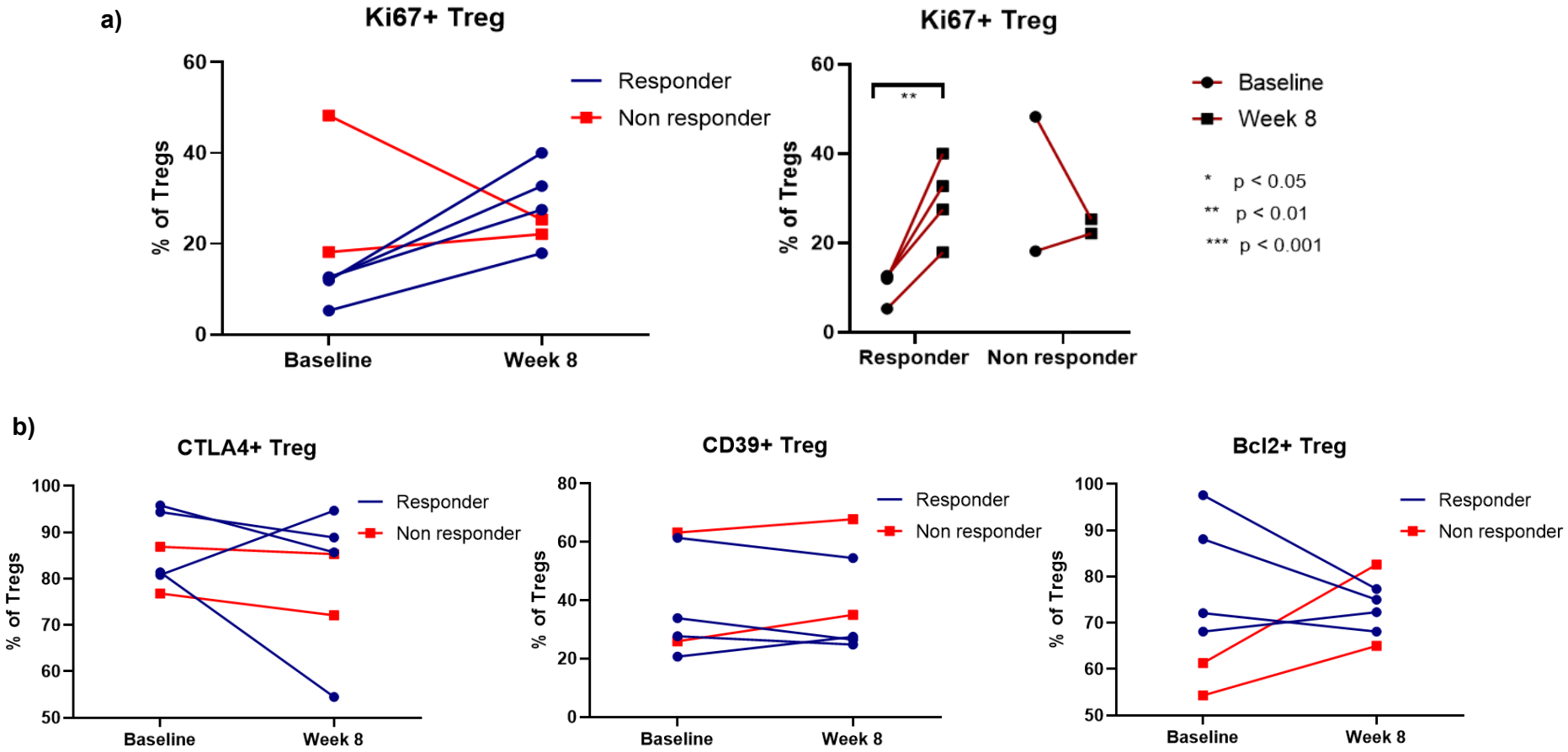


Figure 5 - 15 : Changes in colonic mucosal regulatory CD4 T cell functional marker frequencies following FMT.

a) Line graph demonstrating change in Ki67+ Tregs with subgroup analysis based on clinical response following FMT. b) Line graphs demonstrating non-significant change in other Treg functional markers

5.5.5 Changes in peripheral blood mononuclear cell populations associated with treatment with FMT

No significant change in T cells subsets following FMT

The frequencies of Treg, Th17, Th1 like, Th2 like, CD8 T cells and B cells did not change significantly at week 8 compared to baseline regardless of clinical outcome – Figure 5 - 16.

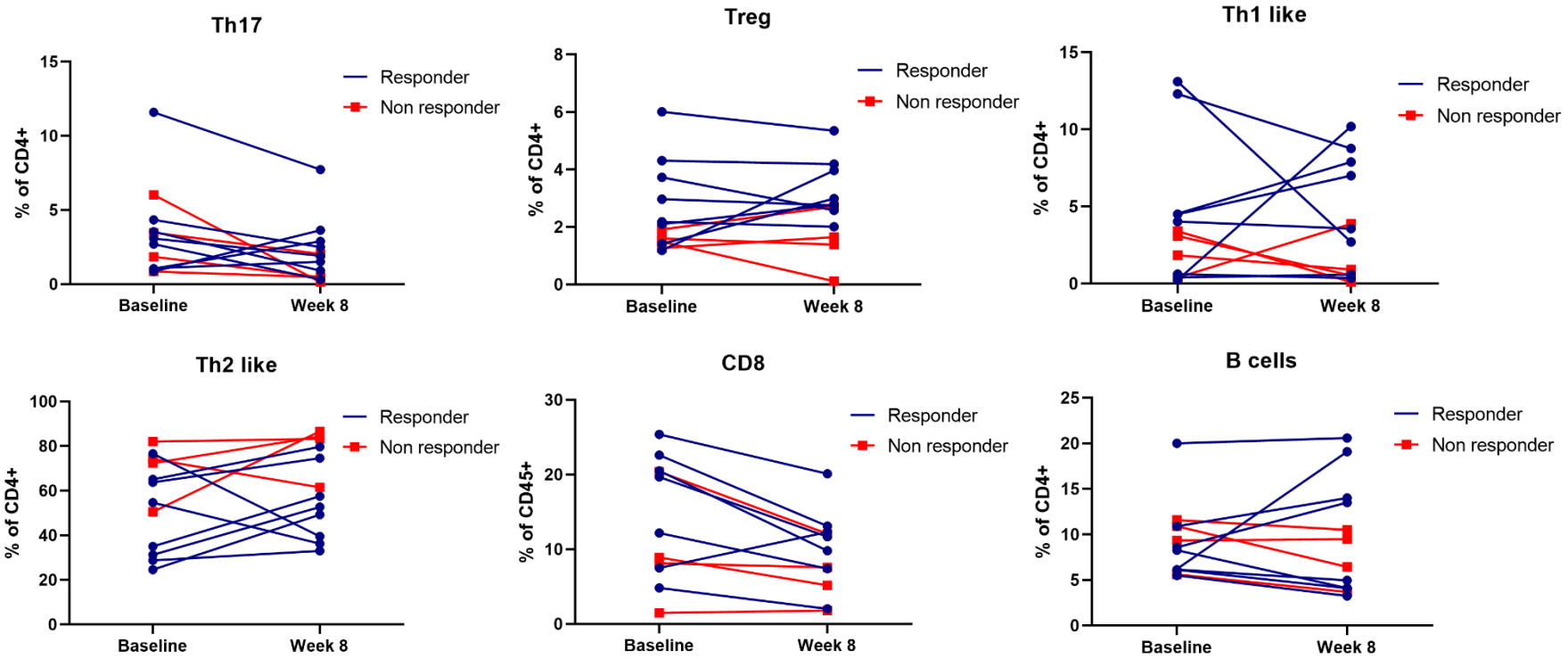
Only IL-10 producing CD4 T cell subsets increase significantly in responders

IL-10 producing CD4 T cells subsets appear to increase significantly in responders following FMT (delta 0.45%; p=0.02). This significant difference was not seen in non-responders and when analysed as a single cohort. No difference was seen in frequencies of IFNy, TNFa, IL-17, IL-5, IL-13 and dual producing IFNy/IL-17 producing CD4 T cells following FMT – Figure 5 - 17.

No significant change in regulatory T cell subsets

The frequencies of naïve Tregs, effector memory and central memory Tregs and gut homing Tregs did not change significantly at week 8 compared to baseline regardless of clinical outcome – Figure 5 - 18.

a)



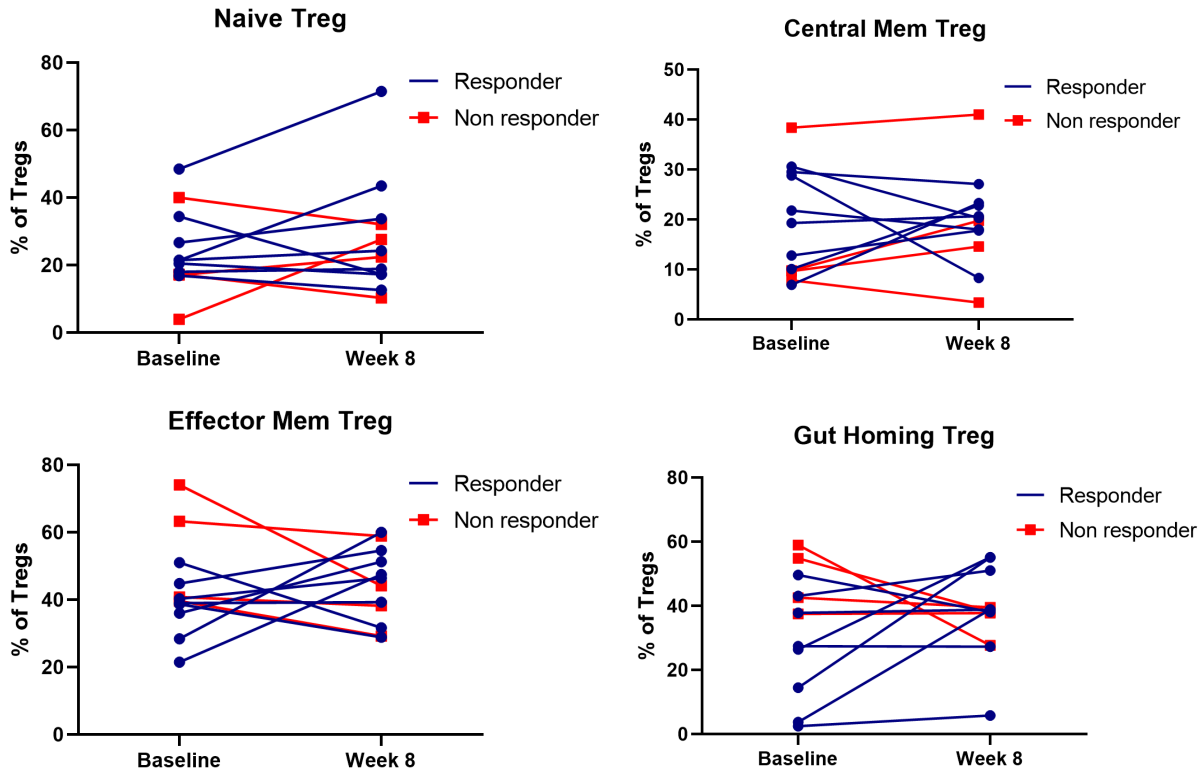
b)

	Δ Week 8 compared to baseline in all patients	Δ Week 8 compared to baseline in responders	Δ Week 8 compared to baseline in non-responders
Treg cells	0.19% (P = 0.576)	0.34% (P = 0.479)	-0.1% (P = 0.84)
Th17 cells	-1.33% (P = 0.08)	-0.86% (P = 0.332)	-2.27% (P = 0.16)
Th1 cells	11.14% (P = 0.94)	17.13% (P = 0.94)	-0.82% (P = 0.63)
Th2 cells	6.62% (P = 0.29)	5.33% (P = 0.52)	9.18% (P = 0.44)
B cells	0.38% (P = 0.79)	1.48% (P = 0.47)	-1.83% (P = 0.16)
CD8 cells	0.05% (P = 0.99)	-2.41% (P = 0.66)	4.99% (P = 0.29)

Figure 5 - 16 : Changes in key peripheral blood mononuclear immune subset frequencies following FMT.

a) Line graph demonstrating non-significant change in key PBMC subsets following FMT. b) Table showing mean change of PBMC subsets at week 8 compared to baseline with subgroup analysis based on clinical response.

a)



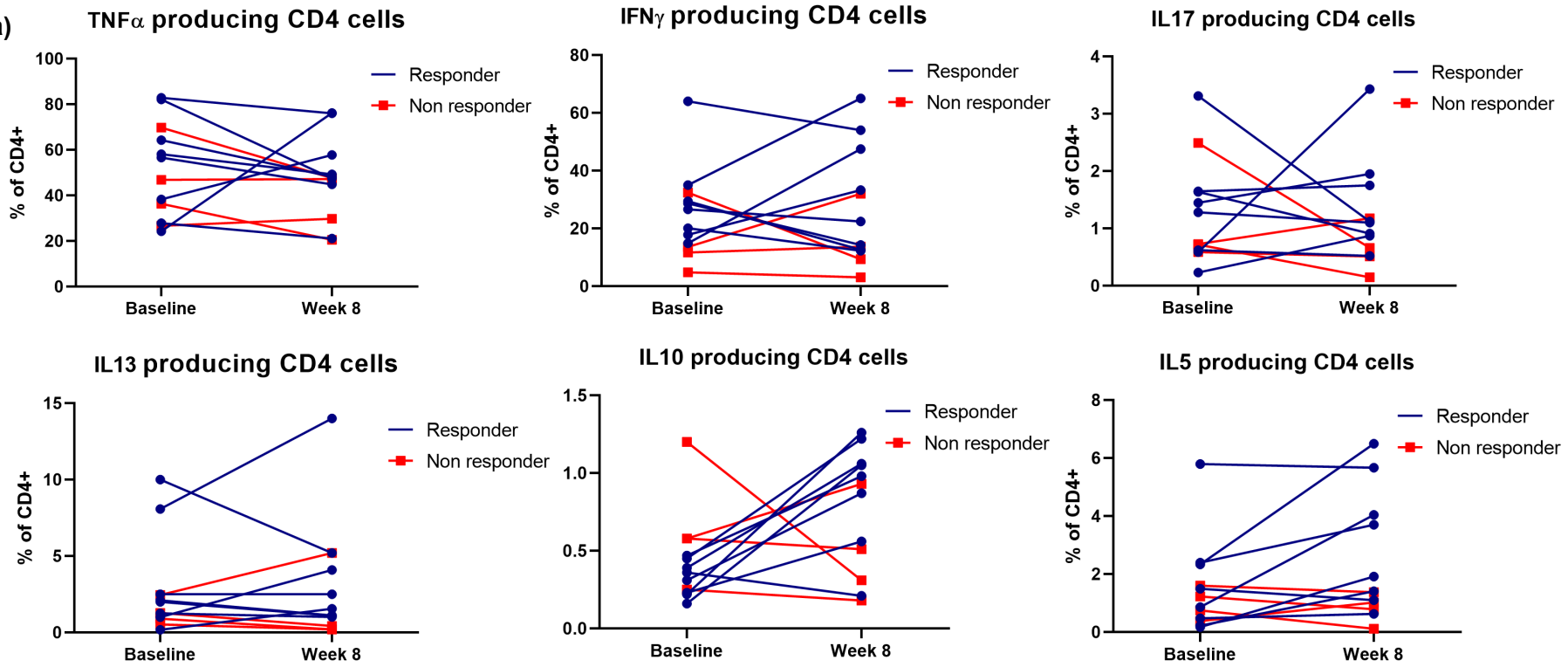
b)

	Δ Week 8 compared to baseline in all patients	Δ Week 8 compared to baseline in responders	Δ Week 8 compared to baseline in non-responders
Naïve Tregs	3.78% (P = 0.34)	3.91% (P = 0.44)	3.52% (P = 0.67)
Central Memory Tregs	0.96% (P = 0.75)	-0.19% (P = 0.97)	3.25% (P = 0.36)
Effector Memory Tregs	1.06% (P = 0.84)	7.5% (P = 0.26)	-11.83% (P = 0.16)
Gut Homing Tregs	4.54% (P = 0.47)	13.14% (P = 0.09)	-12.65% (P = 0.18)

Figure 5 - 17 : Changes in key peripheral blood mononuclear Treg subset frequencies following FMT.

a) Line graph demonstrating non-significant change in key PBMC Treg subsets following FMT. b) Table showing mean change of PBMC Treg subsets at week 8 compared to baseline with subgroup analysis based on clinical response.

a)



b)

	Δ Week 8 compared to baseline in all patients	Δ Week 8 compared to baseline in responders	Δ Week 8 compared to baseline in non-responders
TNF	-4.02% (P = 0.54)	-1.69% (P = 0.86)	-8.68% (P = 0.25)
IFN	1.71% (P = 0.75)	3.11% (P = 0.67)	-1.09% (P = 0.91)
IL17	-0.1% (P = 0.8)	0.11% (P = 0.83)	-0.51% (P = 0.37)
IL10	0.24% (P = 0.14)	0.45% (P = 0.02)	-0.17% (P = 0.56)
IL5	0.8% (P = 0.1)	1.27% (P = 0.07)	-0.16% (P = 0.6)
IL13	0.36% (P = 0.65)	0.44% (P = 0.71)	0.22% (P = 0.81)

Figure 5 - 18 : Changes in peripheral blood mononuclear key cytokine producing immune subset frequencies following FMT.

a) Line graph demonstrating non-significant change in PBMC cytokine producing CD4 subsets following FMT. b) Table showing mean change of PBMC cytokine producing CD4 subsets at week 8 compared to baseline with subgroup analysis based on clinical response. Only IL-10 producing CD4 frequencies increased significantly in responders.

5.6 Transcriptomics

5.6.1 Quality control of 3' RNA-seq reads

An average of 10.2 million 75bp single ended reads were generated per sample after sequencing. Following quality control - adapter removal, poly-A tail removal and filtering of low quality and short read sequences an average of 9.9 million reads remained. These were then used for alignment with the Human Genome database with an average of 72% mapping uniquely and 25% mapping to multiple loci.

5.6.2 Differential gene expression analysis

Paired differential gene expression analysis was undertaken comparing baseline and week 8 transcriptomic datasets. 155 genes were upregulated and 72 were downregulated at week 8 compared to baseline in all 12 patients completing the study as shown in Figure 5 - 19. On subgroup analysis, 135 genes were upregulated and 43 were downregulated at week 8 compared to baseline in clinical responders and 52 genes were upregulated and 14 were downregulated at week 8 compared to baseline in non-responders (Figure 5 - 20 and Figure 5 - 21). In the responders there was an increase in gene associated with metabolic pathways involved in cellular respiration and cellular re-modelling and a decrease in genes associated with anti-microbial peptides, pro-inflammatory cytokines and pro-inflammatory cytokine receptors, cytotoxic mediators including granzyme A, immunoglobulins and microbial binding proteins. Conversely, in the non-responders, there was a significant increase in specific anti-microbial peptides (alpha defensins, S100A, Reg3A), proinflammatory mediators, however a decrease in specific anti-microbial peptide defensin was also observed. On comparing week 8

gene expression data, 54 genes were upregulated and 266 genes were downregulated in responders compared to non-responders as shown in Figure 5 - 22. Genes associated with multiple antimicrobial peptides including Reg family, defensins, S100 proteins along with multiple proinflammatory immune activation pathways were significantly downregulated in responders compared to non-responders.

5.6.3 Gene enrichment pathway analysis

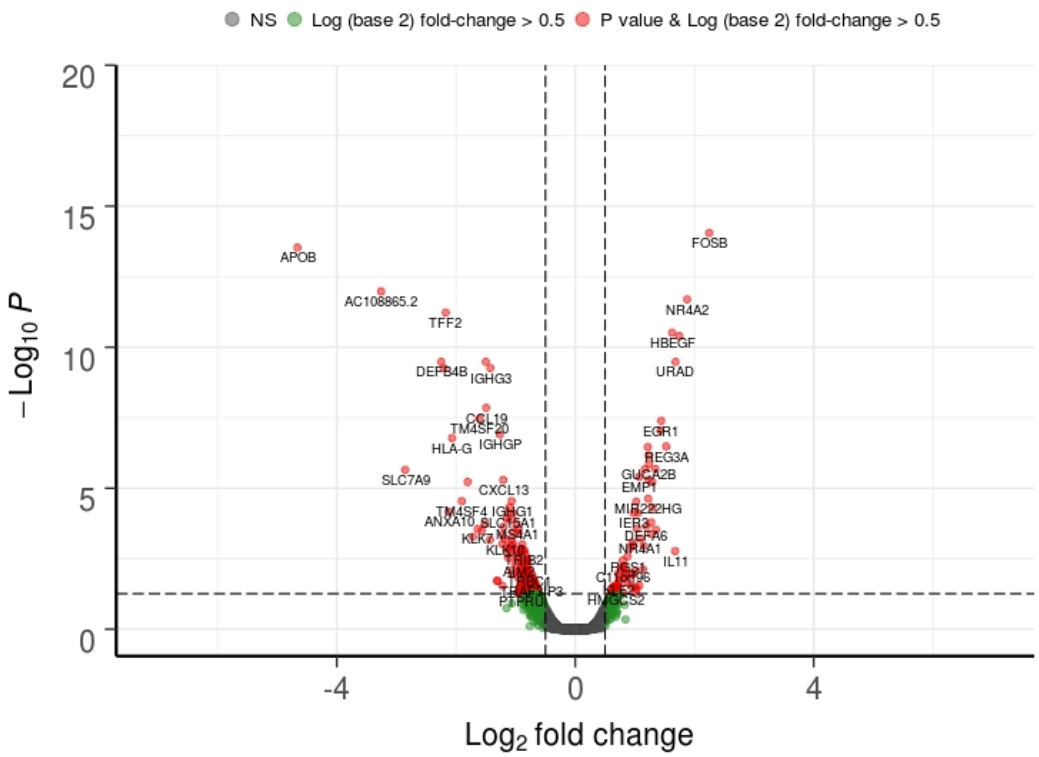
Pathway analysis was performed using the Camera bioinformatics pipeline as described in methodology. Paired analysis comparing baseline and week 8 gene expression datasets revealed differential regulation of 140 gene ontology biological processes pathways and 74 KEGG/Reactome pathways in all patient in the study (Figure 5 - 23). However, on subgroup analysis there was differential regulation of 265 gene ontology biological processes (of which 96 were upregulated and 169 downregulated) and 93 KEGG/Reactome pathways (of which 56 were upregulated and 37 downregulated) in responders (Figure 5 - 24 and Figure 5 - 25). Pathways associated with multiple facets of pro-inflammatory immune activation, microbial defence, antigen binding, inflammatory mediators and leucocyte trafficking were downregulated, and pathways associated with metabolism, cellular respiration and butyrate and propionate metabolism were upregulated in responders.

In contrast, there was differential regulation of 23 gene ontology biological processes (of which 16 were upregulated and 8 downregulated) and 36 KEGG/Reactome pathways (of which 25 were upregulated and 11 downregulated) in non-responders (Figure 5 - 26). Pathways

associated with matrix re-modelling were downregulated and antigen presentation and inflammation were upregulated in this subgroup.

On comparison of week 8 gene expression datasets between responders and non-responders there was differential regulation of 676 gene ontology biological processes (of which 102 were upregulated and 574 downregulated) and 137 KEGG/Reactome pathways (of which 38 were upregulated and 99 downregulated) in non-responders (Figure 5 - 27). Pathways associated with multiple facets of pro-inflammatory immune activation, microbial defence, antigen binding, inflammatory mediators and granulocyte migration, leucocyte trafficking were downregulated, and pathways associated with metabolism, cellular respiration and butyrate and propionate metabolism were highly upregulated in responders compared to non-responders.

Upregulated	Downregulated	No differential expression
155	72	12,453



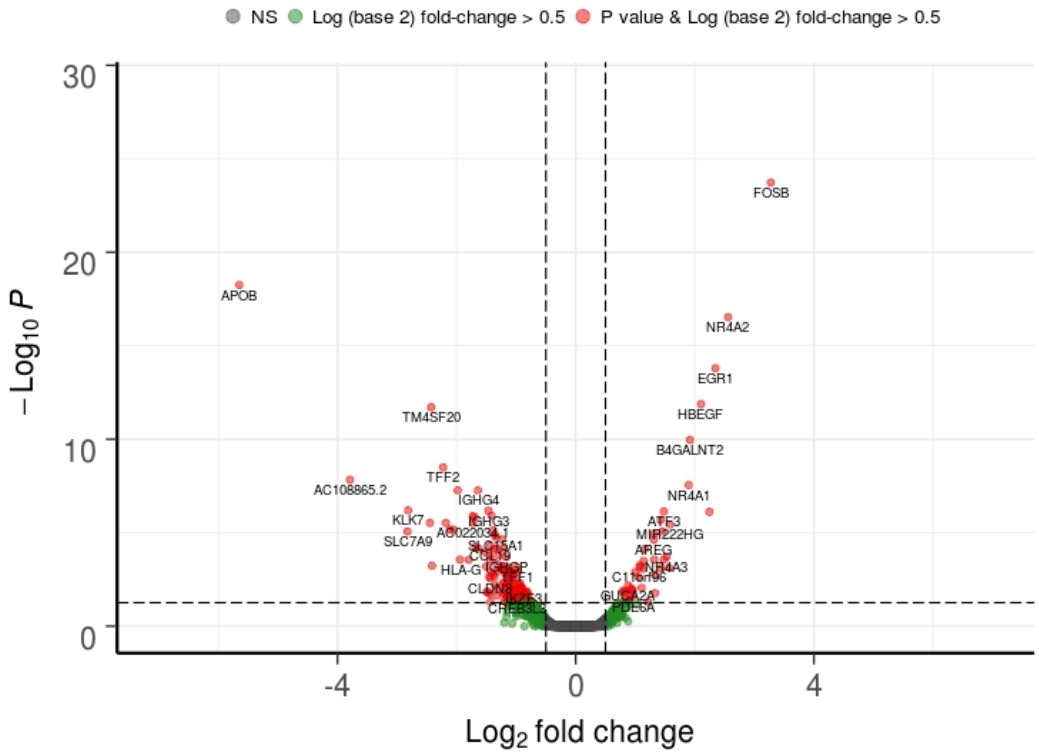
Upregulated		
Genes	**logFC**	**FDR**
FOSB	2.25	8.94E-15
NR4A2	1.88	2.01E-12
B4GALNT2	1.75	4.06E-11
URAD	1.68	3.31E-10
IL11	1.68	1.68E-03
HBEGF	1.63	3.04E-11
REG3A	1.53	3.29E-07
EGR1	1.44	4.08E-08
AQP8	1.43	9.02E-08
ITLN2	1.35	3.00E-04
DEFA5	1.35	2.10E-06
OTOP2	1.29	6.02E-06
NR4A3	1.29	4.85E-05
EYA2	1.28	1.63E-04
REG1B	1.27	4.20E-04
GUCA2B	1.24	1.40E-06
AREG	1.23	6.93E-07
PCK1	1.23	5.03E-06
MIR222HG	1.23	2.37E-05
ARL14	1.22	3.44E-07

Downregulated		
Genes	**logFC**	**FDR**
APOB	-4.66	2.92E-14
AC108865.2	-3.25	1.05E-12
SLC7A9	-2.85	2.24E-06
DEFB4B	-2.25	3.31E-10
DEFB4A	-2.21	5.47E-10
TFF2	-2.17	5.92E-12
ANXA10	-2.12	6.87E-05
HLA-G	-2.07	1.65E-07
TM4SF4	-1.9	2.87E-05
CLDN8	-1.8	6.02E-06
HOXD13	-1.72	5.30E-04
KLK7	-1.64	2.80E-04
TM4SF20	-1.61	3.48E-08
PNLIPRP2	-1.57	3.27E-04
SERPINB7	-1.52	1.88E-04
IGHG4	-1.5	3.31E-10
CCL19	-1.49	1.41E-08
AC119673.2	-1.43	6.71E-04
IGHG3	-1.42	5.47E-10
CYP3A4	-1.31	1.91E-02

Figure 5 - 19 : Differential gene expression profiles and volcano plots between baseline and week 8 in all participants

Volcano plots demonstrating log2 fold change of genes against negative log10 P value. Top 20 upregulated and downregulated differentially expressed genes listed in table. Full list available as supplementary table.

Upregulated	Downregulated	No differential expression
135	43	12,515



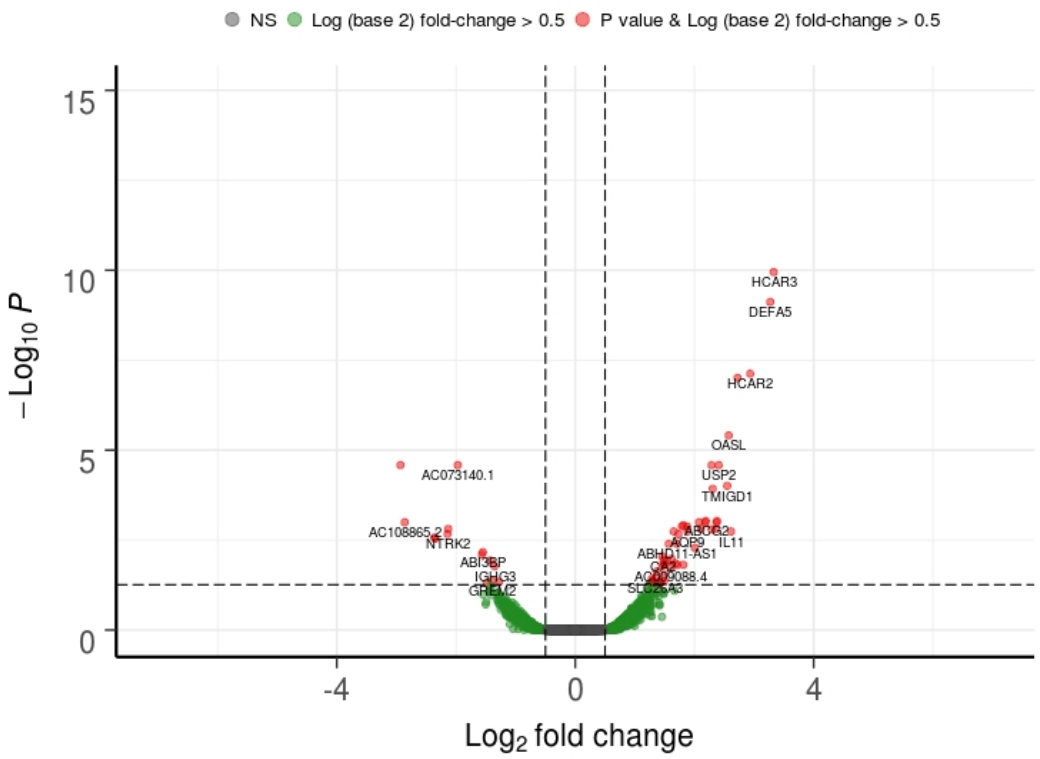
Upregulated		
Genes	logFC	FDR
FOSB	3.28	2.01E-24
NR4A2	2.56	3.20E-17
EGR1	2.35	1.69E-14
LINC00923	2.25	7.81E-07
HBEGF	2.1	1.39E-12
B4GALNT2	1.92	1.12E-10
NR4A1	1.9	2.84E-08
MIR222HG	1.58	3.64E-06
ASPG	1.57	7.71E-04
NR4A3	1.52	1.92E-04
ATF3	1.48	7.62E-07
EGR3	1.48	2.75E-04
URAD	1.45	8.75E-06
FOS	1.43	2.11E-06
ACKR3	1.43	7.71E-04
ITLN2	1.34	1.73E-02
MOCS1	1.33	1.82E-03
PCK1	1.32	1.20E-05
ADAMTS1	1.32	2.75E-04
AREG	1.31	2.41E-05

Downregulated		
Genes	logFC	FDR
APOB	-5.65	5.86E-19
AC108865.2	-3.79	1.49E-08
SLC7A9	-2.82	8.75E-06
KLK7	-2.81	6.49E-07
TM4SF4	-2.45	3.05E-06
TM4SF20	-2.43	2.03E-12
ANXA10	-2.41	5.94E-04
TFF2	-2.23	3.29E-09
DEFB4B	-2.18	3.05E-06
DEFB4A	-2.11	6.89E-06
TCN1	-2.05	7.04E-06
S100A8	-1.98	5.56E-08
HLA-G	-1.94	2.75E-04
CYP3A4	-1.79	2.84E-04
GLDN	-1.73	1.31E-06
AC022034.1	-1.72	3.05E-06
S100A9	-1.7	1.50E-06
KLK10	-1.68	5.87E-05
IGHG4	-1.64	5.56E-08
SAA1	-1.63	7.23E-05

Figure 5 - 20 : Differential gene expression profiles and volcano plots between baseline and week 8 in responders

Volcano plots demonstrating log2 fold change of genes against negative log10 P value. Top 20 upregulated and downregulated differentially expressed genes listed in table. Full list available as supplementary table.

Upregulated	Downregulated	No differential expression
52	14	12,631



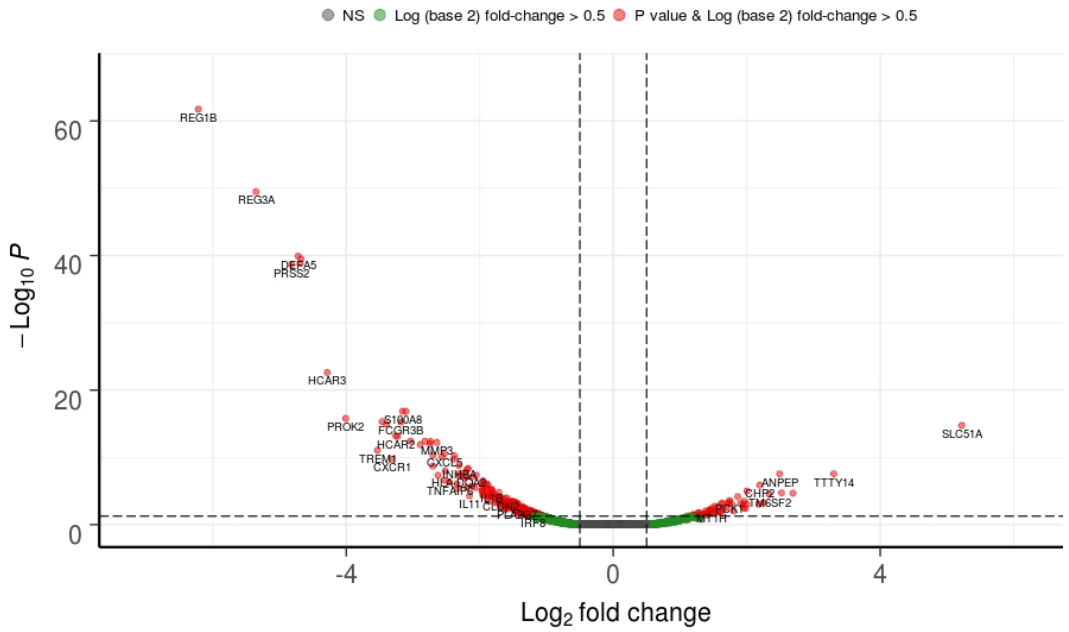
Upregulated		
Genes	logFC	FDR
HCAR3	3.33	1.13E-10
DEFA5	3.27	7.61E-10
HCAR2	2.94	7.50E-08
REG3A	2.72	9.87E-08
IL11	2.61	1.82E-03
OASL	2.57	3.91E-06
TMIGD1	2.55	9.86E-05
USP2	2.41	2.61E-05
SLC30A10	2.38	9.27E-04
CLDN23	2.37	1.01E-03
AQP8	2.36	1.55E-03
REG1A	2.31	1.19E-04
DEFA6	2.29	2.61E-05
OTOP2	2.29	1.58E-03
ABCG2	2.19	9.27E-04
URAD	2.18	1.01E-03
PLAAT2	2.11	1.55E-03
REG1B	2.08	1.01E-03
GUCA2B	2.01	5.21E-03
AQP9	1.88	1.82E-03

Downregulated		
Genes	logFC	FDR
HOXD11	-2.93	2.61E-05
AC108865.2	-2.86	1.01E-03
DEFB4A	-2.36	2.65E-03
DEFB4B	-2.35	2.88E-03
NTRK2	-2.14	2.16E-03
AC119673.2	-2.13	1.55E-03
AC073140.1	-1.97	2.61E-05
CCL19	-1.57	7.94E-03
ABI3BP	-1.55	6.87E-03
INSL5	-1.49	4.84E-02
IGHGP	-1.43	1.16E-02
GREM2	-1.39	4.27E-02
IGHG3	-1.36	1.69E-02
IGHG4	-1.28	4.60E-02

Figure 5 - 21 : Differential gene expression profiles and volcano plots between baseline and week 8 in non-responders

Volcano plots demonstrating log2 fold change of genes against negative log10 P value. Top 20 upregulated and downregulated differentially expressed genes listed in table. Full list available as supplementary table.

Upregulated	Downregulated	No differential expression
54	266	11,609

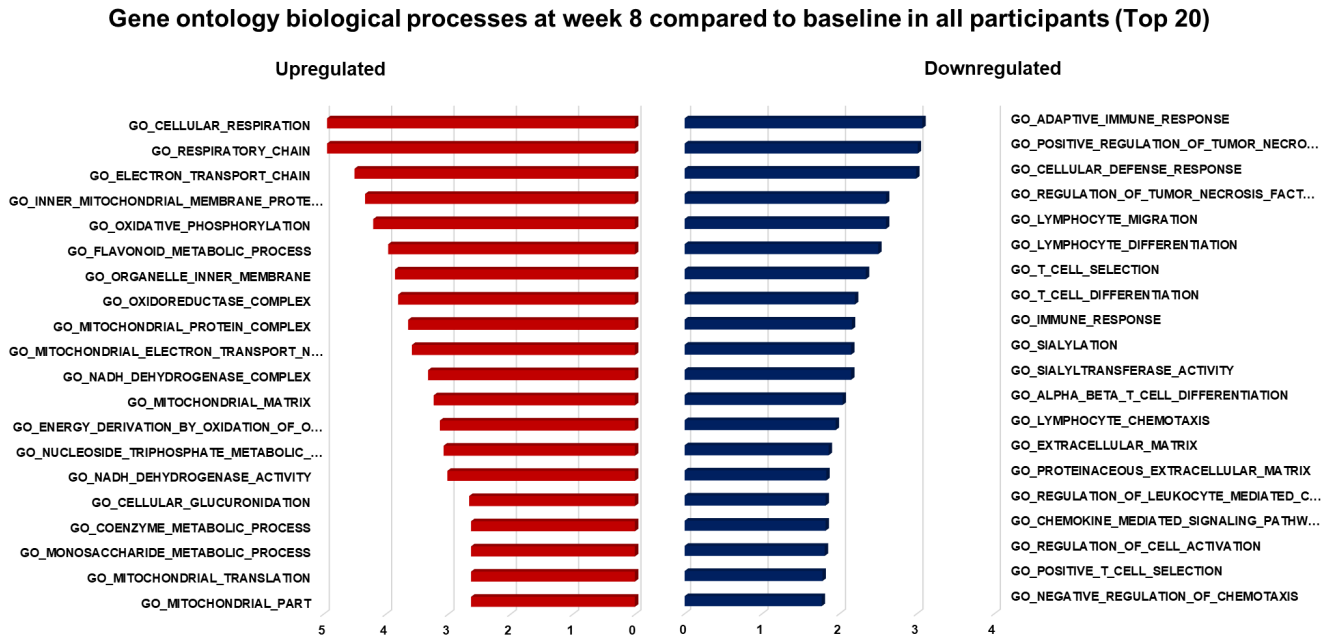


Upregulated		
Genes	logFC	FDR
SLC51A	5.22	1.84E-15
TTTY14	3.31	2.71E-08
DIO3OS	2.69	2.14E-05
DIO3	2.52	1.92E-05
ANPEP	2.49	2.79E-08
TM6SF2	2.34	3.04E-05
LINC00278	2.25	6.05E-04
CHP2	2.2	1.46E-06
PRORY	2.18	9.26E-04
AQP8	2	1.02E-05
HOXD11	2	9.26E-04
AC010086.3	1.98	3.79E-03
GBA3	1.96	3.25E-04
SERPINB3	1.95	3.31E-03
HOXD10	1.95	1.07E-03
SCUBE2	1.91	7.01E-04
ADH1C	1.87	6.87E-05
SLC13A2	1.8	9.32E-03
HHIP	1.78	1.01E-03
PCK1	1.74	2.58E-04

Downregulated		
Genes	logFC	FDR
REG1B	-6.22	1.78E-62
REG3A	-5.35	3.39E-50
PRSS2	-4.82	2.28E-39
DEFA5	-4.72	1.23E-40
DEFA6	-4.69	1.55E-39
REG1A	-4.68	3.22E-40
HCAR3	-4.28	2.48E-23
PROK2	-4.01	1.67E-16
TREM1	-3.53	8.36E-12
CXCR2	-3.46	4.76E-16
SPP1	-3.4	1.18E-15
CXCR1	-3.31	1.88E-10
HCAR2	-3.26	6.45E-14
AQP9	-3.23	7.88E-14
FCGR3B	-3.18	5.90E-16
S100A8	-3.16	1.49E-17
S100A9	-3.11	1.49E-17
FPR1	-3.04	3.84E-13
CXCL8	-2.89	1.33E-12
G0S2	-2.82	4.18E-13

Figure 5 - 22 : Differential gene expression profiles and volcano plots between responders and non-responders at week 8

Volcano plots demonstrating log2 fold change of genes against negative log10 P value. Top 20 upregulated and downregulated differentially expressed genes listed in table. Full list available as supplementary table.



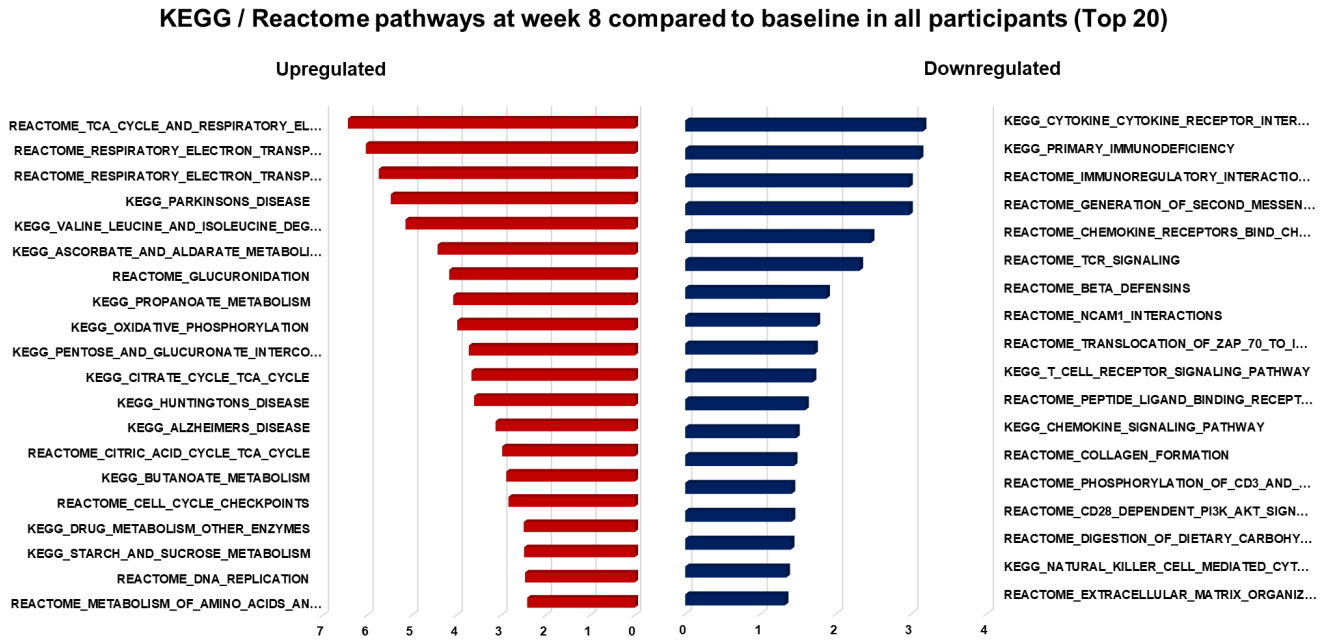


Figure 5 - 23 : Gene ontology biological processes and KEGG/Reactome pathways at week 8 compared to baseline in all participants.

Top 20 gene ontology biological processes and KEGG/Reactome pathways by competitive gene set testing using Camera.

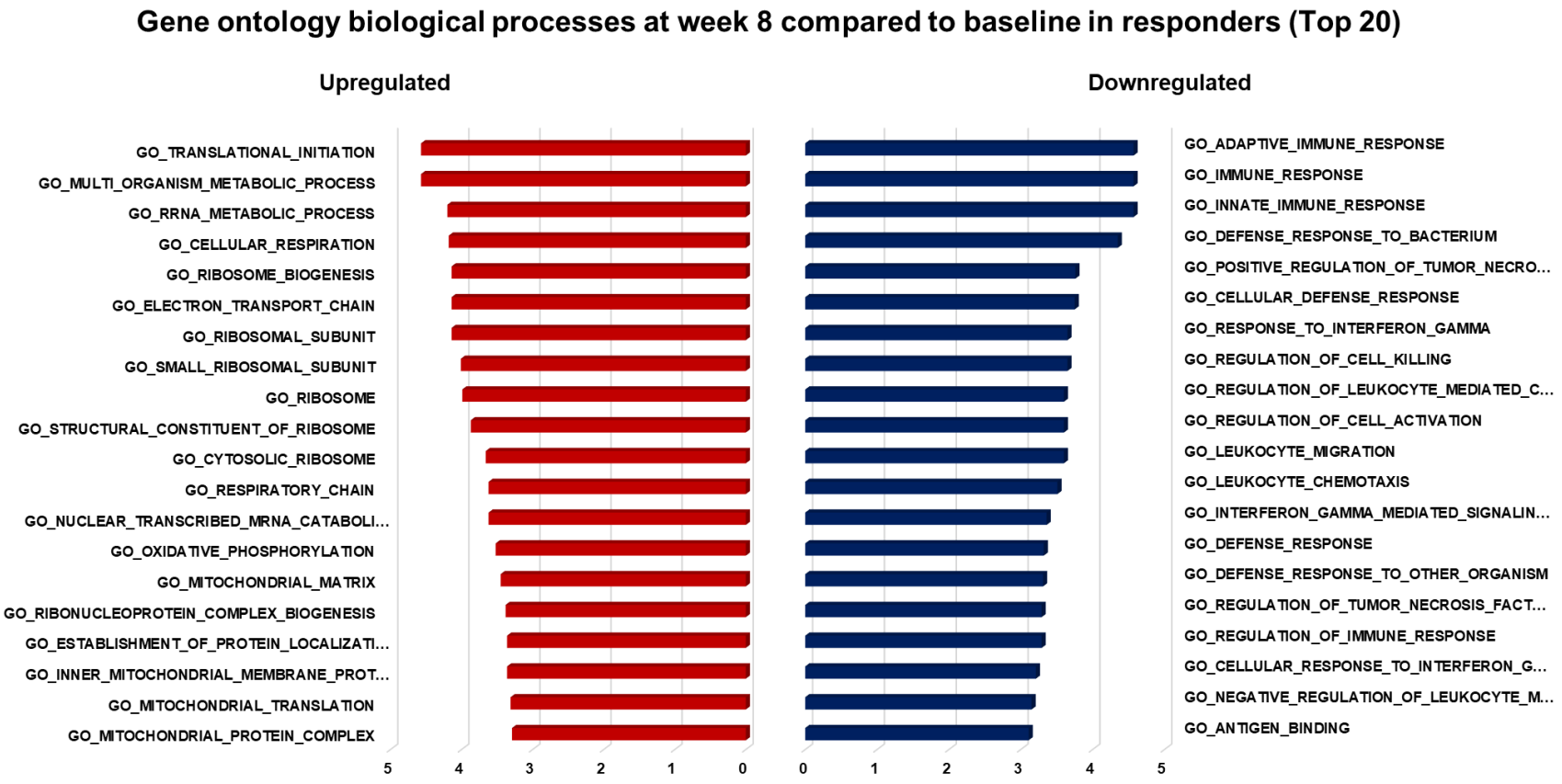


Figure 5 - 24 : Gene ontology biological processes at week 8 compared to baseline in responders.

Top 20 gene ontology biological processes by competitive gene set testing using Camera.

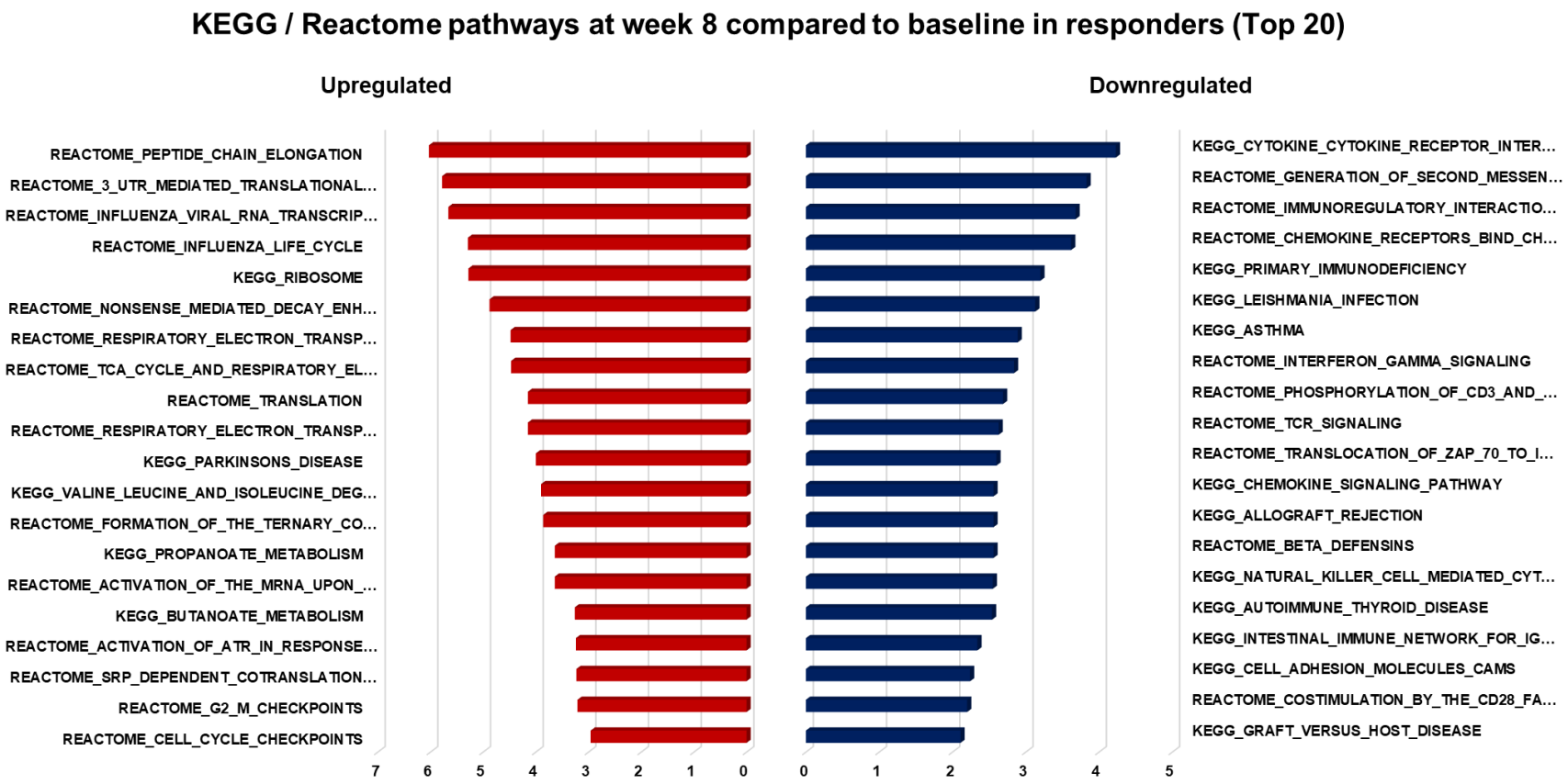
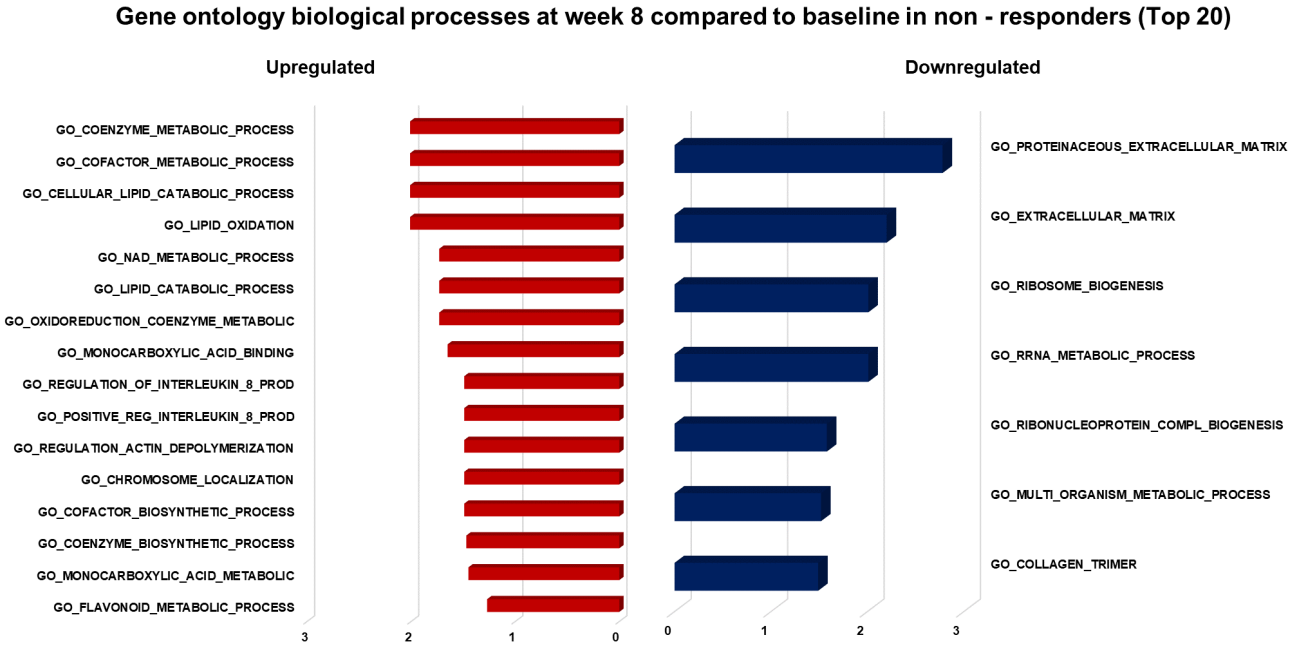


Figure 5 - 25 : KEGG/Reactome pathways at week 8 compared to baseline in responders.

Top 20 KEGG/Reactome pathways by competitive gene set testing using Camera.



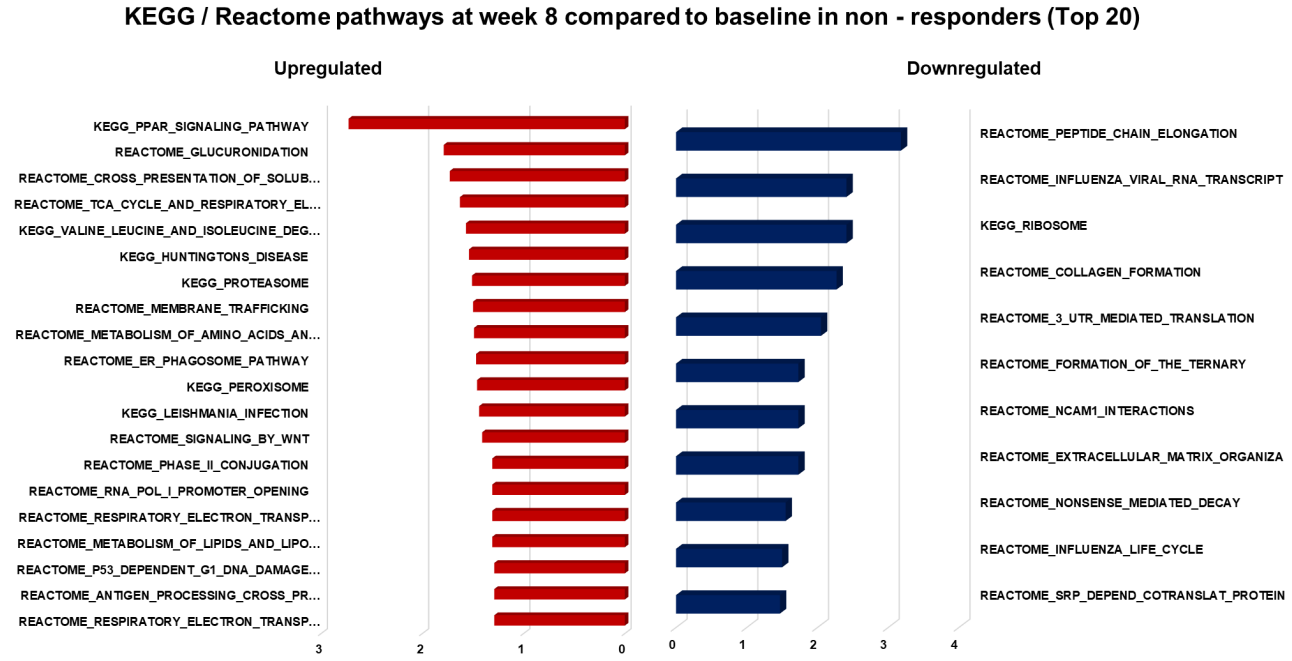
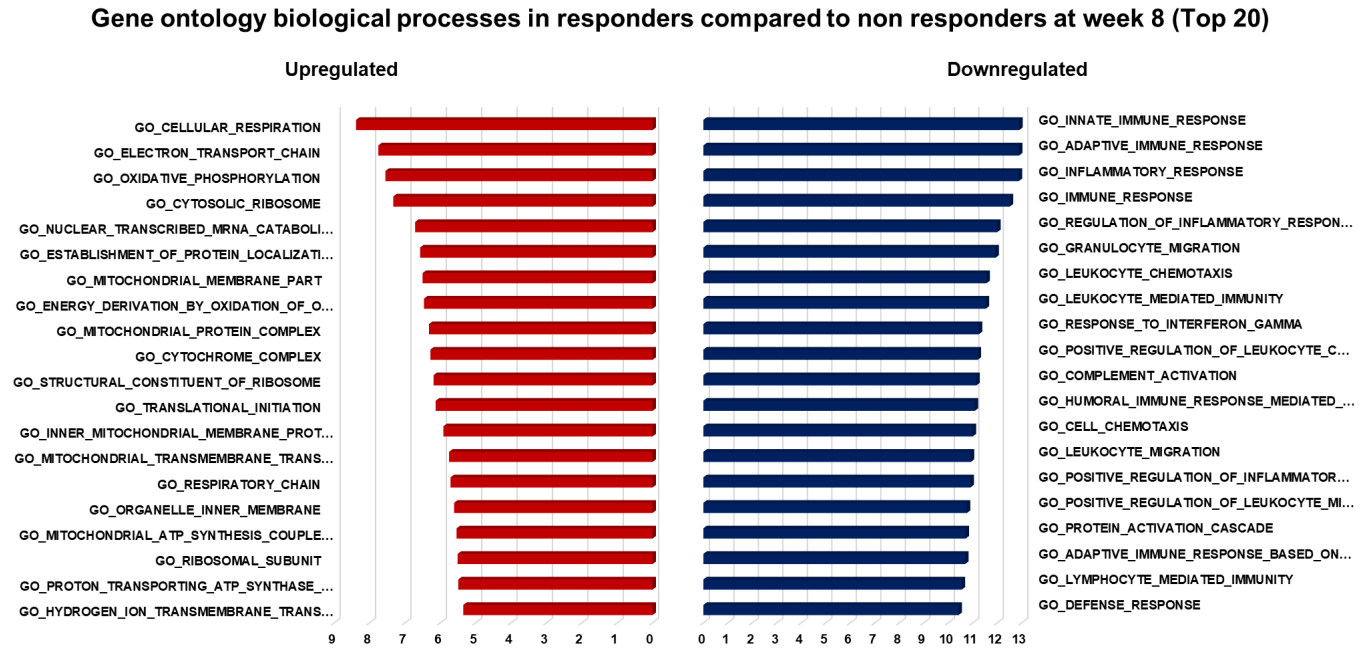


Figure 5 - 26 : Gene ontology biological processes and KEGG/Reactome pathways at week 8 compared to baseline in non - responders.

Top 20 gene ontology biological processes and KEGG/Reactome pathways by competitive gene set testing using Camera.



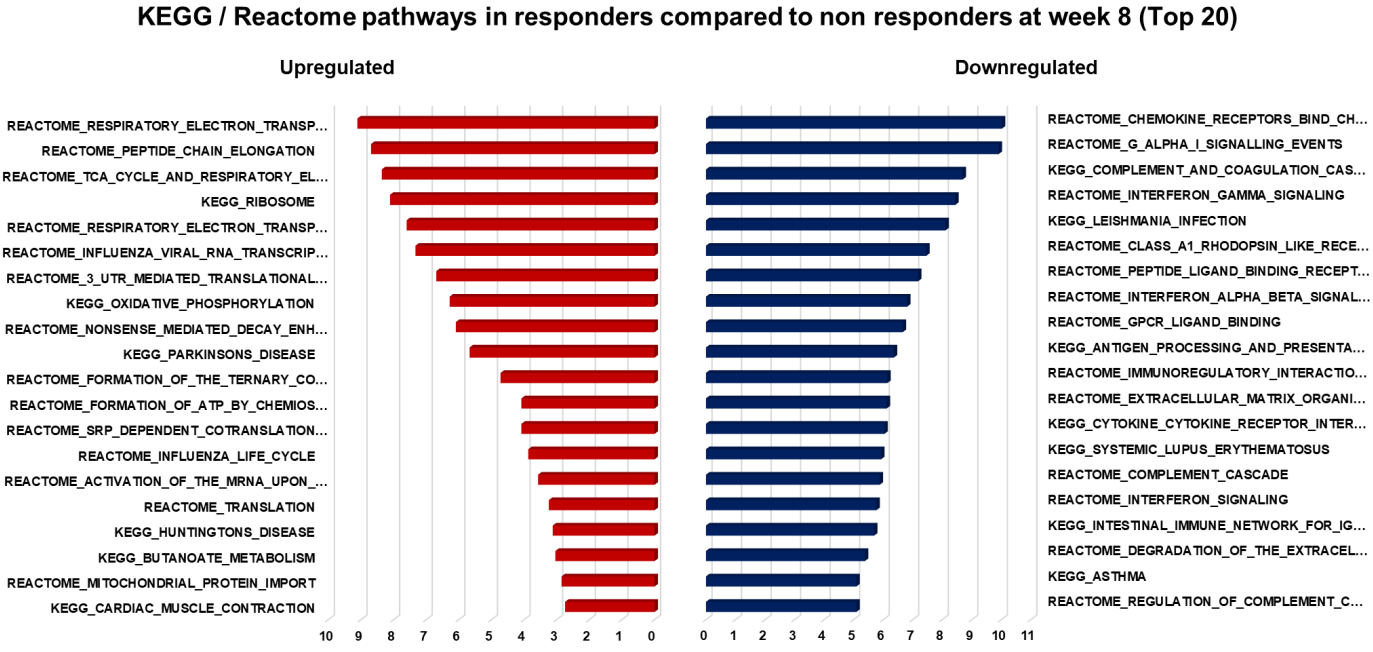


Figure 5 - 27 : Gene ontology biological processes and KEGG/Reactome pathways in responders compared to non – responders at week 8.

Top 20 gene ontology biological processes and KEGG/Reactome pathways by competitive gene set testing using Camera.

5.9 Discussion

The STOP-Colitis pilot study is the first investigation to date exploring differences between nasogastric and colonic delivery of FMT on tolerability and potentially efficacy in the treatment of active ulcerative colitis. This chapter presents clinical data from the patient cohort recruited at Birmingham along with mechanistic findings in changes in host biology and gut microbiota. The main clinical findings of this sub-study was that FMT possibly appeared to be more effective and better tolerated when given by the colonic route compared to via a nasogastric tube. As this is a sub-study and does not present data from the entire cohort, these findings need to be interpreted with caution, especially since the study (and this sub-study) was not powered to demonstrate efficacy.

5.9.1 Clinical outcomes

Nevertheless, keeping in mind limitations related with an open label study, FMT resulted in an overall clinical remission rate of 41% and clinical response rate of 47% based on an intention to treat analysis. This was higher at 58.3% and 66.7% respectively when a per protocol analysis was performed. Endoscopic and histological remission and was reached in 23.5% with no differences between route of delivery. We also noted that consistent with other phase 3 clinical trials in IBD, biologic experienced patients had a slightly lower rate of clinical remission and response (37.5%) compared to biologic naïve patients.

In comparison, a meta-analysis of RCTs demonstrated that clinical remission was achieved in 39 of 140 (28%) patients in the donor FMT groups (compared with 13 of 137 (9%) patients in the placebo groups) and clinical response was achieved in 69 of 140 (49%) donor FMT patients (compared to 38 of 137 (28%) placebo patients) (254, 255). In the cohort studies, 39

of 168 (24%; 95% CI: 11%-40%) achieved clinical remission. The recently published RCT of the use of FMT in active UC by Costello et al showed a more comparable remission rate of 32% (12 of the 38 participants) and a clinical response rate of 55% (21/38) (127).

It is however important to note that none of these studies are directly comparable. Although all have used FMT as the intervention for treatment active ulcerative colitis, there is a very significant heterogeneity in nearly all likely confounding elements of the study (Table 1 - 1). These include variability in the manufacturing process / production of FMT (aerobic versus anaerobic), number of infusions delivered (ranged from 1 to 40), differences in definition of clinical remission, differences in patient inclusion criteria and route of delivery of FMT (nasogastric versus colonic). These differences in both product (FMT) design and study design is the lack of rationalised standardisation of FMT due to the paucity of research exploring differences in protocols. This is also limited by the knowledge that the active constituent(s) or its target(s) are unknown and is likely to differ between diseases.

5.9.2 Time to clinical remission and response

One of the challenges in the field of therapeutic use of FMT is identifying the number of FMT treatment sets / infusions needed to induce the clinical outcome of interest; which in the case of ulcerative colitis is resolution of active disease as assessed by clinical remission and response. Consequently, defining number of FMT infusions needed in clinical trials has been arbitrary and based on rational ‘best estimates’. In our current FMT delivery protocol found that the overall mean time to sustained clinical remission was 4 weeks (95% CI 5.31 – 2.69) and the overall mean time to clinical response was 1.63 weeks (95% CI 2.11 – 1.14). This did not appear to be significantly different based on route of delivery, however it’s important to note that the study was not powered to assess this and the FMT infusion protocols

differed between colonic and nasogastric routes of delivery. Nevertheless, these early signals suggest that clinical response appears to be fairly rapid. All patients who achieved clinical response by week 4 either went on to achieve clinical remission or maintain clinical response by week 8 and week 12. Similarly, all patients who failed to respond to FMT by week 4 did not progress to achieve clinical response or clinical remission by week 8 or week 12. This is in contrast to the multi-donor FMT RCT conducted by Paramsothy and colleagues, where the initial FMT was delivered by colonoscopy followed by enemas 5 days per week for 8 weeks (252). Here they demonstrated that at week 4 the clinical response was significantly greater in patients allocated faecal microbiota transplantation than in those assigned placebo (17/41 [41%] vs 5/40 [13%] of 40; RR 3.3, 95% CI 1·4–8.1; p=0.003). However, the clinical remission did not differ significantly between study groups at this time point (12/41 [29%] vs 5/40 [13%] respectively; RR 2.3, 95% CI 0.9–6.0; p=0.064). Time to remission or response was not assessed in other studies.

5.9.3 Acceptability and tolerability

A critical outcome from the STOP-Colitis pilot was the tolerability of FMT based on the route given. In the Birmingham cohort, 4 patients withdrew early from the study due to lack of tolerability of the nasogastric tube. There is vast global and local experience with FMT delivered via the nasogastric route for the treatment of recurrent and refractory Clostridium difficile infection and to date there have been no problems reported with regards to tolerability. However unlike for this indication where only a single infusion is needed in patients (who quite often may already have an NG tube in situ for enteral feeding during critical illness), our protocol for STOP-Colitis required patients to have an NG tube for 8 days. Nearly all patients who were screened and recruited in to the study conveyed their apprehension right from the outset of having a nasogastric tube inserted if randomised to that route of delivery, more so than any unpleasantness associated with having FMT. It was

therefore not unexpected that the early termination rate due lack of tolerability was higher in this arm of the study. Consequently, one patient withdrew from the study before having had any FMT administered due to and one patient could only have one infusion as they refused to have the NG tube reinserted.

5.9.4 Changes in host biology

This sub-study of STOP-Colitis is the first to date to take a focused approach in investigating changes in the host biological response to FMT for the treatment of UC. By identifying key changes in colonic mucosal immune cell populations and changes in the colonic transcriptomic landscape we have been able to build on current evidence from animal studies in understanding biological mechanisms that determine success in response to FMT. Furthermore, by linking these to changes in the gut microbiota, we are able to explore microbial mediated pathways that are potentially determine these host responses. Importantly, these findings also help us get closer to understanding causal microbial mechanisms to development and progression of IBD.

5.9.5 Immunological changes associated with FMT

Colonic inflammation in IBD is associated with a loss of adequate immunomodulatory function with a concurrent increase in pro-inflammatory pathways. The mechanisms that lead to and are associated with this loss of immune homeostatic control remains unknown. There is, however, increasing evidence to suggest that the dysregulated gut microbiome in IBD is likely to contribute to this.

Changes in colonic lamina propria immune cell subsets

In my analysis of colonic mucosal lamina propria mononuclear cells, I identified a significant increase in the immunomodulatory FoxP3+ regulatory T cells and a reduction in the proinflammatory Th17 cell population frequencies following treatment of active UC with FMT. These shifts were significantly greater and only significant in the patients who had a clinical response to FMT in comparison to the non-responders. Intestinal Foxp3+ Tregs exert their immunosuppressive activity, at least in part, through production of the cytokine IL-10. Consistent with these findings, the population frequencies of IL-10 producing CD4 T cells significantly increased and IL-17 producing CD4 T cells significantly deceased with the shift again being significant and greater in clinical responders. These findings tend to strongly suggest that the abrogation of colonic inflammation that results from treatment with FMT is associated with a significant functional increase in the immunosuppressive mechanisms mediated by regulatory T cells and IL-10 producing CD4 cells (including Tregs) and reduction in the Th17 and IL-17 induced pro-inflammatory pathways.

Colonic Tregs are largely developed against microbial antigens and like other non-lymphoid tissues (256), Tregs are enriched in intestinal lamina propria with around 25-35% harbouring in the colon. Gnotobiotic / germ free mice that lack any microbiota, have significantly lower number of colonic Tregs compared to specific pathogen-free (SPF) and wild type mice (257, 258). There are two primary two sources of Treg cells in the body: thymus derived natural Treg cells (tTreg or nTreg) are generated in the thymus early in life and peripheral Treg (pTreg or iTreg) that are generated in various tissues of the body throughout life (259, 260). The thymus is a major site for the induction of Treg cells, where FoxP3 expression is initiated by a combination of influences from the microenvironmental and recognition of antigens (259, 261). However, FoxP3 negative thymic generated Tregs can however be peripherally induced to express FoxP3 in tissues such as the colon and placenta where

immunoregulatory function is needed to maintain homeostasis (262). tTregs are generated following interaction of high-affinity T-cell receptors with MHC class II molecules presenting self-antigens in order to supress autoimmune responses(263). pTregs develop from naïve CD4+ T cells following binding of microbial antigens and dietary signal molecules (specifically protein antigens) with their TCR and stimulation with TGF-β and retinoic acid (264). Adoptive transfer of naïve T-cells from the transgenic lines made from colonic TCRs resulted in very efficient conversion to peripherally induced Tregs (pTregs) in mesenteric LNs and colon (265). While pTregs are largely accepted to be the primary source of intestinal Treg population, under experimental conditions where generation of extra-thymic Tregs is compromised, tTregs can migrate to the gut lamina propria and proliferate to fill up the niche (266).

The findings of an increase in that colonic Treg population in clinical responders to FMT raise the question of its source / origin and function. In the pivotal work by Atarashi et al, they discovered that a substantial fraction of Foxp3+ Tregs observed in SPF and Clostridium-colonized mice were negative for Helios - a transcription factor expressed in thymus-derived natural tTregs (102, 248, 267). This appears to suggest that the increase in Tregs associated with colonisation of Clostridia species were likely to be iTregs possibly induced as a result of specific microbiota, microbial antigens and metabolites in the colon. Furthermore, by using IL-10 reporter mice they demonstrated that introduction of these Clostridia strains resulted in a similar induction of IL-10 expressing Foxp3+ Tregs to those discerned in SPF mice and a much higher population compared to germ free mice. The recently reported seminal study by Britton and colleagues demonstrated that gut microbiome transplantation from individuals with IBD into germfree mice induced colitis and promoted a decrease in Tregs with concurrent expansion of Th2 and Th17 populations (105). In contrast, transplantation of stool from healthy individuals into germ-free mice resulted in the development of anti-inflammatory IL-10 producing Tregs along with suppression of Th17

activity by inhibition of NF-kB signalling. These observations appear to indicate that the specific gut bacteria and their metabolites provide a signal for the peripheral induction of IL-10+ Tregs in colonic lamina propria resulting in suppression of inflammation. Furthermore, I observed that baseline Th17 cell populations appeared to be higher and baseline Treg populations were lower in responders compared to non-responders, this was not statistically significant. It could be postulated that patients with a dominant microbiota mediated baseline immunological imbalance are more likely to respond to FMT compared to those whose colonic inflammation is dominated by other inflammatory triggers.

Tregs have been shown to occupy their own homeostatic compartmentalised niche such as within secondary lymphoid tissue in the gut. This presence of significant populations of Tregs in multiple lymphoid and non-lymphoid organs suggests that these Tregs in different tissues are possibly maintained by distinct homeostatic mechanisms (261). Tissue-resident Tregs may have different gene expression profiles or functions compared with their counterparts in lymphoid organs or TGF-B induced Tregs – pTregs (268). This suggests a complex mode of tissue-specific regulation of Tregs by Foxp3 and co-factors such as the surround colonic environment with microbial and dietary products that control differentiation, maintenance, and function. Other than based on the site of induction, pTregs can also be broadly classified into two separate populations due to their distinct homeostatic behaviours: CD45RA-CCR7+ central memory Tregs (central Tregs) and CD45RA-CCR7- effector memory Tregs (effector Tregs) (269). Although central Tregs are quiescent and express high-levels of anti-apoptotic molecules such as Bcl-2, they recirculate through the secondary lymphoid tissues by upregulating lymphoid homing molecules CCR7 and CD62L (270, 271). Effector Tregs, on the other hand, are highly proliferative, plastic, prone to apoptosis due to decreased expression of Bcl-2 and Mcl-1 and downregulate lymphoid homing molecules. As a result, effector Tregs are the dominant Treg population in nonlymphoid tissues and are largely

tissue-resident demonstrating distinct phenotypic and functional profiles in response to different microenvironments (261).

Once these effector Tregs are generated in lymph nodes, they need to home to the gut to undergo local expansion and consequently mediate immunological tolerance. This is achieved by expression of gut-homing receptors such as the chemokine receptor CCR9 and the integrin α4β7, which allow their migration to the lamina propria (272). Regulatory T cell patrol of secondary lymphoid organs is not sufficient to maintain immunological tolerance and therefore, specialisation into discrete tissue-resident subsets is needed. The importance of the role of gut homing Tregs in colonic mucosal immunosuppressive effects is highlighted by experiments that have demonstrated abrogation of tolerance in mice with gut-homing defects, despite regular generation of FoxP3-expressing cells in the lymph nodes (273, 274). Furthermore, immune tolerance needs to be sustained locally in the intestinal lamina propria and cannot be fully established at the time of T cell activation in the lymph nodes.

Changes in colonic mucosal functional Treg subsets following FMT

In order to explore the origin of the increase in colonic mucosal regulatory T cells following FMT, I characterised changes in these specific Treg subsets. I did not seek to discriminate changes between peripheral and thymic Treg cells as distinguishing very low numbers of Helios+ FoxP3+ tTregs would have been very challenging and error prone through standard flow cytometry. Characterisation of differences in Treg populations however convincingly demonstrated that there was a significant increase in effector Treg populations following FMT. On subgroup analysis, this difference was greater and only significant in patients who had responded to FMT. Furthermore, consistent with these findings there was a significant increase in gut homing effector colonic mucosal Treg populations based on α4 expression and this again was strongly associated with clinical response to FMT. Analysis of Tregs

based on functional markers in a subset of patients revealed that the expression of the proliferation-associated nuclear Ki67 increased significantly following FMT in responders. This finding is consistent with expansion of effector Tregs in the secondary lymphoid tissues confirming a likely mechanism for this increase in gut homing effector Tregs. There was no change in central Tregs and naïve Tregs following FMT. Moreover, there was no change in other functional markers including anti-apoptotic CTLA-4, survival protein Bcl-2 and CD39 however the small sample size for comparisons limits its interpretation.

Collectively these novel findings are crucial in understanding immunological mechanisms by which FMT is associated with modulation of colonic inflammation in UC. Following FMT, specific mediators (microbial, metabolic and / or dietary) activate conventional T cells or naïve Tregs either directly or via interaction with dendritic cells in the lamina propria and mesenteric lymph nodes in order to promote conversion to effector Tregs. These Tregs cells induced in mesenteric lymph nodes express gut-homing receptors to facilitate their migration to the colonic lamina propria. Here they are able to induce immunosuppressive effects, which mediated by production of IL-10, reduce proinflammatory Th17 cell and IL17 production potentially through activation of STAT3 induced downregulation of pro-inflammatory gene expression (275).

Effector Tregs, particularly those with a gut homing profile, are thought to have encountered antigens more recently than central Tregs cells and consequently through enhanced migration through non-lymphoid tissues are able to exert local immunosuppressive effects (261). This brings into question which key factors in FMT are involved to induce this differentiation. As mentioned previously, studies in germ-free mice have demonstrated that bacterial commensals are crucial for the development of sufficient colonic Treg numbers with bacteria including *Bacteriodes fragilis* and Clostridium species being essential for intestinal

Treg homeostasis (85). Furthermore, these studies have importantly shown that the SCFAs propionate, butyrate and acetate are able to independently restore colonic Treg cell numbers in germ-free or antibiotic-treated mice and increase numbers in SPF mice (247). Although many effector Treg characteristics are similar to those of antigen-experienced conventional T cells, it remains uncertain if direct T cell receptor ligation with microbial antigens alone is sufficient to drive Treg cell differentiation or whether other stimuli are needed.

No changes in peripheral blood immune cell subsets following FMT

I did not find any significant differences in key immune subset and Treg subset frequencies on characterisation of peripheral blood mononuclear cells apart from a slight increase in IL-10 producing CD4 T cells following FMT following FMT. These findings are similar to those described by Costello et al where no significant differences were seen in PBMCs CD4 subsets following FMT regardless of clinical response (127). This is not unexpected especially in view of the fact that immune cell homeostasis is generally compartmentalised. Therefore, the effects of local treatment with FMT to the gastrointestinal tract are highly unlikely to have a systemic effect. Furthermore, there was a relatively lower proportion of Tregs and Th17 cell population frequencies in PBMC compared to those in the colonic lamina propria, and this is consistent with literature.

5.9.6 Changes in colonic mucosal transcriptome following FMT

Exploration of changes in the gene expression profiles of colonic mucosa has provided valuable insights into the biological mechanisms of efficacy of FMT for the treatment of active UC. Multiple genes and pathways associated with microbial defence mechanisms, MHC II antigen presentation, immune activation and proteases were significantly downregulated following FMT. This was more pronounced in clinical responders and absent

or upregulated in non-responders. Furthermore, responders had a significant downregulation of these mechanisms and pathways at week 8 when compared to non-responders at the same time point. In contrast, genes associated with short chain fatty acid metabolism (butyrate and propionate) and cellular respiration and remodelling were significantly upregulated in responders.

Response to FMT is associated with downregulation of anti-microbial defence mechanisms

These findings are fascinating as they provide both useful insights into mechanisms of FMT and potentially mechanisms that underlie development of IBD. A multitude of anti-microbial peptides including beta-defensins, regenerating family member (REG) family, S100A were downregulated in responders both when compared to baseline as well as in comparison to week 8 data from non-responders. Antimicrobial peptides are conservative tools of the innate immune system that provide an immediate response to a large set of various pathobionts. These peptides are widely expressed by most epithelial cells and released at epithelial surfaces and in a site of inflammation. They are also stored in granules of phagocytic cells and exert their effects in phagolysosomes or by being extracellularly secreted. AMPs act on the bacterial cell wall either directly or indirectly by forming nets to prevent bacterial invasion. Most important in the gut epithelium are α-defensins produced by Paneth cells and ß-defensins produced by the colonic epithelium. These are further complemented in the Paneth cell by lysozymes, lectins in particular from the REG family and cathelicidins such as human cathelicidin antimicrobial peptide (CAMP). One of the key findings identified through studies in mucosal immunology in IBD is the upregulation of anti-microbial peptides. The REG and defensin family of genes were found to be significantly upregulated in treatment naïve UC compared to healthy controls (181). Expression of antimicrobial peptide genes has been shown to be significantly elevated in both inflamed as well and non-inflamed tissues of patients with Crohn’s disease when compared to healthy

controls suggesting that expression of these peptides are independent of endoscopic evidence on an inflammatory state (205).

Following FMT, a significant reduction in ß-defensins was seen in responders with ß-defensin-4 (hBD-4) showing the greatest change. Patients with ulcerative colitis have been shown to have significantly higher levels of hBD-4 gene expression in the colon compared to healthy controls (276, 277). hBD-4 is induced by microbial pathogens that gain access to the epithelial surface where they interact with TLRs which consequently activate defensin expression. Expression of other ß-defensins has been observed by exposing epithelial cells to different bacteria (276, 278, 279). In addition to its bactericidal role, hBD-4 has been shown to act as a chemotactic to monocytes (280). Although changes in the gut microbiota have not been analysed as part of this thesis, prior studies in UC have shown that a shift in gut microbiota away from the aberrant IBD baseline following FMT has been associated with remission. Thereby a reduction in ß-defensins following FMT maybe hypothesised to be an immunological consequence of a reduced abundance of suspected pathobionts that are associated with mucosal invasion and a proinflammatory state.

The antimicrobial REG proteins are secreted C-type lectins that are predominantly expressed in the gastrointestinal tract. REG are up-regulated upon bacterial colonization of the gut as well as during enteric infection and inflammation (58, 281). They have bactericidal activity against both Gram positive and Gram-negative bacteria and contribute to the spatial separation of intestinal bacteria and the epithelium. Gut microbiota are in closer contact to intestinal epithelial cells in the absence of REG peptides (56, 282). Activation of TLR in intestinal epithelial cells and Paneth cells has been shown to stimulate expression of REG3G and REG3B in these cells (281, 283). Altering the IBD gut dysbiosis as a

consequence of administration of FMT appears to significantly dampen production of REG class of proteins.

Interestingly the S100 class of proteins were found to be downregulated in responders. The proteins S100A8 and S100A9 form a heterodimer called calprotectin - a calcium and zinc-binding protein which constitutes 60% of cytosolic proteins in neutrophils. Expression of calprotectin inhibits bacterial binding to mucosal epithelial cells and plays a protective role in innate host defence (284). Calprotectin expression has shown to reduce epithelial invasion by pathogens, including *Listeria* and *Salmonella*. Granulocytes produce calprotectin at sites of mucosal inflammation in the colon and is used for both to aid diagnosis and monitoring of disease in IBD (285). Faecal calprotectin levels have shown to have a good correlation with the degree of inflammatory activity in IBD (286). A reduction of S100 class of proteins following FMT is therefore likely to confirm a decrease in the mucosal inflammation and possibly abrogation of anti-microbial response.

Reduction in anti-microbial peptides were even more striking in responders when compared to non-responders at week 8. Both defensins (alpha and beta) and REG class of peptides were significantly reduced in responders. Interestingly, both defensins and S100A8 were upregulated in DSS-colitis mice following gavage of FMT from normobiotic mice (139). This is likely to represent a limitation associated with non-immunological animal models of IBD in studying host-microbiota interactions.

Response to FMT is associated with upregulation of SCFA metabolic pathways

Pathways in responders to FMT at week 8 compared to baseline, in addition to downregulation of anti-microbial and pro-inflammatory immunological pathways, were

characterised by upregulation of pathways associated butanoate and propanoate metabolism. These were not seen in non-responders to FMT. Furthermore, butanoate metabolism was significantly upregulated, and antigen presentation downregulated in responders when compared to non-responders at week 8. Butyrate and propanoate are short chain fatty acids (SCFA) produced by the intestinal bacteria by the fermentation of nondigestible polysaccharides. A presumption can be made that the increase in SCFA metabolic pathways seen in responders are likely to be a consequence of an increase in SCFA producing bacteria following FMT. This would be in keeping with data from the previous RCTs that have demonstrated that clinical response to FMT in patients with active UC was associated with an increase in abundances of species belonging to *Clostridium* clusters IV, XIVa and XVII such as *Roseburia intestinalis*, *Ruminococcaceae* and *Lachnospiraceae* (137, 138). These bacteria produce SCFA in the gut and would be consistent with the transcriptomic changes associated with SCFA metabolic pathways following FMT. Importantly, this is also likely to correspond and shed light on the mechanisms that drive the increase in regulatory T cells following FMT (explained further in the next section). It would be imperative to confirm these findings by corroborating the SCFA measurements before and after FMT and its correlation with host gene expression and abundances of SCFA producing bacteria (based on metagenomics) through a multi-omics integration approach. SCFA and gut microbiome data from the trial was unfortunately not available at the time of submission of this thesis.

Based on this study it would be challenging to resolve if the differences gene expression following FMT are a consequence of the treatment or just a marker of clinical response. Prior studies have shown differences in the colonic mucosal transcriptomic landscape between inflamed and non-inflamed tissue. One study demonstrated a difference in 6 genes only in a cohort of patients with Crohn's disease (205). None of the genes or pathways were associated with anti-microbial defence response. A study investigating colonic mucosal gene

expression of patients with UC demonstrated that genes that included the REG family, S100 and defensins were significantly upregulated in both patients with active and inactive UC compared to non-IBD controls. Of these genes, expression of only REG1A was downregulated in the colonic mucosa of patients in remission compared to those with active UC. The study concluded that the involved intestinal mucosa of UC patients does not return to “normal” after inflammation has resolved, but rather remains in a permanently altered state characterised by a unique transcriptional signature (287). Consequently, it may be argued that the changes seen in gene expression, in particular those associated with a significant downregulation of antimicrobial peptides following FMT, are likely to be specific to the treatment rather than a clinical state of remission. This potentially highlights that aberrant gut microbiota in patients with UC is associated with triggering a pro-inflammatory anti-microbial defence response as well as the mechanisms through which FMT from healthy donors appears to moderate this response. Future studies should include control patients in remission induced by other means such as corticosteroids for comparison.

5.9.7 Restoring immunological homeostasis in ulcerative colitis through induction of regulatory T cells

An increasingly recognised feature of gut commensals is their ability to induce Tregs via various molecular mechanisms (Figure 5 -19). Tregs play a critical role towards maintaining peripheral tolerance *in vivo* by actively suppressing proinflammatory T cells cytokine secretion and proliferation. Thus, these cells are critical towards maintaining immune balance and restricting aberrant inflammation. It is likely that introduction or enrichment of specific strains of bacteria through FMT promotes Treg proliferation in the colonic mucosa and lymph nodes, thereby attenuating inflammation (199). Tregs can be induced through products of bacterial metabolism including SCFA, tryptophan metabolites and polysaccharide A (PSA).

SCFA such as acetate (C2), propionate (C3) and butyrate (C4) and organic acids (lactate) are derived from the fermentation of host-indigestible polysaccharide and oligosaccharide along with certain amino acids (200). The metabolism of these saccharides involves enzymatic pathways that are facilitated by several bacterial species. The transcription factor FoxP3 is centrally involved in the establishment and maintenance of the Treg cell phenotype and its activity is regulated post-translationally by histone/protein acetyltransferases and histone/protein deacetylases (HDACs) (288). SCFAs produced by these bacteria are involved in stimulating colonic Tregs through direct physical inhibition of HDACs or inhibition of HDACs through specific G-protein-coupled receptors signalling including GPR41, GPR43 and GPR109a (289). Furthermore, SCFA promotes differentiation of naïve CD4 T cells towards a FoxP3 CD4 cell phenotype. Murine and cell culture studies have demonstrated the direct effect of SCFAs and SCFA producing bacterial species in inducing intestinal Tregs specifically, with higher levels of FoxP3 and IL-10 expression (290, 291). Additionally SCFAs suppress the LPS and cytokine-stimulated production of pro-inflammatory mediators including TNF-α, IL-6 and nitric oxide (NO) (292).

As described earlier, the gut microbiota – butyrate – Treg axis has been explored in seminal work conducted by Atarashi and colleagues (102, 103). They found that a consortium of 46 Clostridium species was able to significantly raise the baseline depressed levels of regulatory T cell populations in germ free mice to the levels observed in conventionally raised mice. Interestingly the Tregs induced by these Clostridium species were Helios negative IL-10 expressing iTregs suggesting peripheral rather than thymic induction of these immunoregulatory cells. Although they demonstrated that Treg differentiation was directly influenced by epithelial cell derived TGF- β1 and indoleamine 2,3-dioxygenase, specific microbe associated molecular patterns were not identified. Furthermore, oral inoculation of Clostridium of conventionally raised mice during the early life resulted in resistance to colitis.

Furusawa and colleagues subsequently demonstrated that colonic luminal concentrations of SCFA positively correlated with colonic Treg abundances (209). The differentiation of Treg cells, both in vitro and in vivo, was induced by butyrate and ameliorated the development of colitis induced in an adoptive T cell transfer model of Rag1(-/-) mice. They identified enhanced histone H3 acetylation in the promoter and conserved non-coding sequence regions of the Foxp3 locus of naive T cells in presence of butyrate suggesting a possible mechanism for microbial-derived butyrate induced differentiation of Tregs. These findings, together with microbiota outcomes from studies of FMT in UC highlight the role of commensal Clostridia as being leading players in the maintenance of gut homeostasis. Phase 1 clinical studies towards exploring the efficacy of human commensal Clostridia strains for the treatment of IBD are currently in progress (193).

Polysaccharide A (PSA), a glycoantigen derived from *Bacteroides fragilis*, has been shown to induce IL-10 producing Tregs via plasmacytoid DCs stimulation in the gut (293, 294). In the absence of APCs this pathway is also mediated by direct interaction of PSA with TLR2 expressed by FoxP3+ Treg cells to stimulate expression of immunoregulatory molecules including IL-10, TGF-β2, and granzyme B (295). Additionally, exposure to PSA induced differentiation of human peripheral CD4+ T cells into type-Tr1 Tregs (FoxP3-) and exhibited nonspecific IL-10 mediated bystander suppression. Intriguingly glycoantigen exposure provoked expression of gut homing receptors on their surface to facilitate gut localization of anti-inflammatory and regulatory responses (296). Furthermore, gut homing of IL-10 producing regulatory T cells and differentiation of naïve CD4 T cells towards a Treg phenotype is induced by interaction of vitamin A metabolite all-trans retinoic acid generated by dendritic cells in presence of microbial antigens (297). However, the underlying mechanisms dictated by gut microbiota in modulating intestinal retinoic acid is unclear. Peroxisome proliferator activated receptor gamma (PPARγ) activation has been shown to drive a Treg phenotype (298). The gut commensal *Bacteroides thetaiotaomicron* exerts its

anti-inflammatory effects by regulating the nuclear-cytoplasmic shuttling of PPAR-γ thereby influencing the immune regulatory landscape in the intestine (299). Finally microbial tryptophan metabolites (kynurenines) produced by bacteria such as *Bifidobacterium longum*, *Bacteroides thetaiotaomicron* and *Faecalibacterium prausnitzii* activate the AhR pathway to reprogram intraepithelial CD4+ T helper cells into immunoregulatory T cells and inhibit polarization towards a pro-inflammatory Th17 phenotype (300-302).

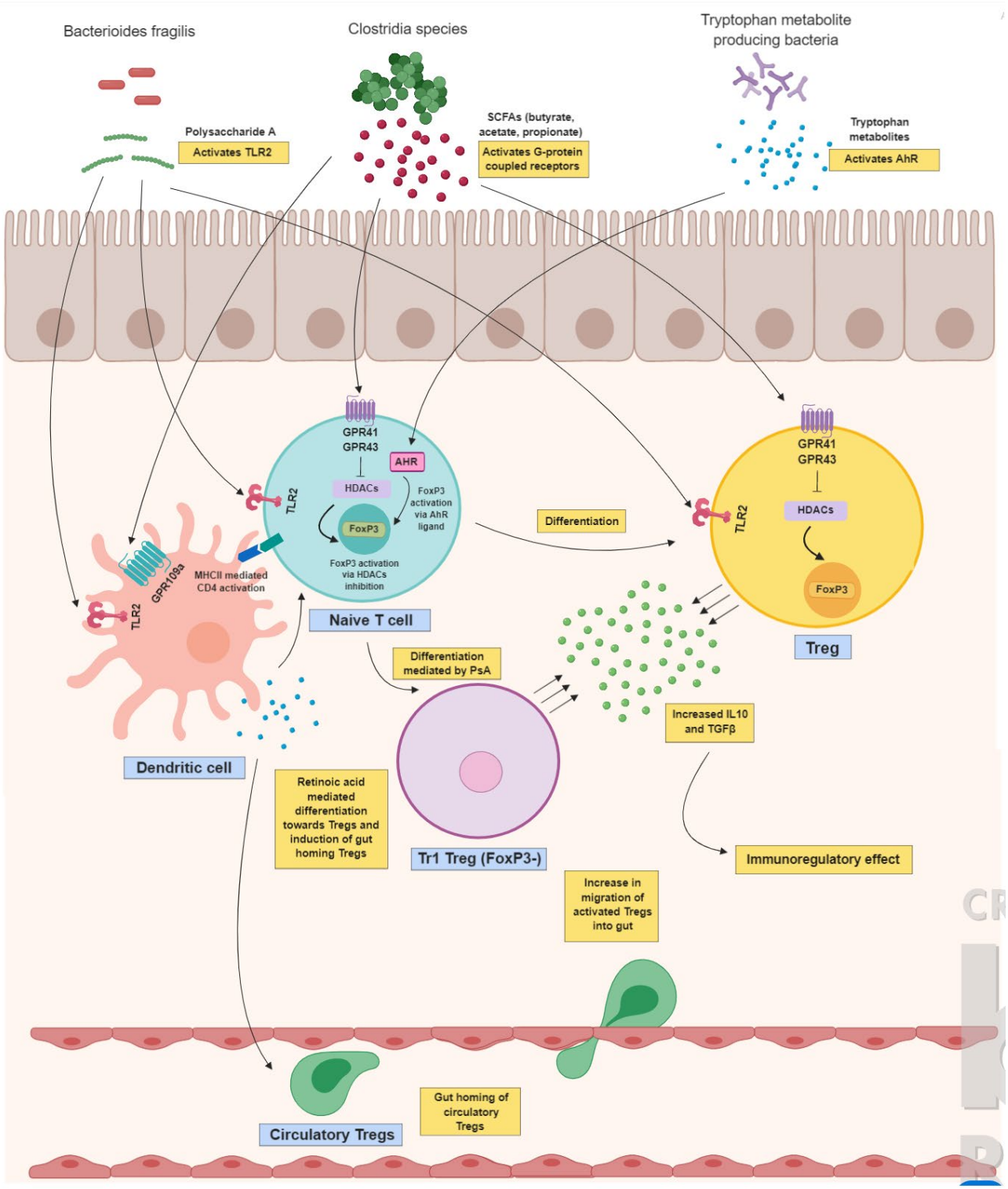

Bacterioides fragilis
Clostridia species
Tryptophan metabolite producing bacteria
Polysaccharide A
Activates TLR2
SCFAs (butyrate, acetate, propionate)
Activates G-protein coupled receptors
Tryptophan metabolites
Activates AhR
GPR41
GPR43
AHR
HDACs
FoxP3 activation via AhR ligand
FoxP3
FoxP3 activation via HDACs inhibition
TLR2
Naive T cell
Differentiation
GPR41
GPR43
HDACs
TLR2
FoxP3
Treg
GPR109a
TLR2
MHCII mediated CD4 activation
Dendritic cell
Differentiation mediated by PsA
Increased IL10 and TGFβ
Retinoic acid mediated differentiation towards Tregs and induction of gut homing Tregs
Tr1 Treg (FoxP3-)
Immunoregulatory effect
Increase in migration of activated Tregs into gut
Gut homing of circulatory Tregs
Circulatory Tregs

Figure 5 - 28 : Likely immunoregulatory mechanisms mediated by gut microbiota as a consequence of FMT in IBD

Microbiota-derived enzymatic activities generate metabolites such as short chain fatty acids (SCFA), and kynurenines. SCFA is involved in stimulating colonic Tregs and differentiation of naïve CD4 T cells through inhibition of HDACs via specific G-protein-coupled receptors signalling. This results in increased histone acetylation in the FoxP3 gene along with an increased Foxp3 expression. Kynurenines (tryptophan metabolites) activate the AhR pathway to polarise naive T cells into FoxP3+ Tregs. Bacteroides fragilis derived polysaccharide A (PSA), induces differentiation of naive CD4 T cells towards IL-10 producing Tregs or into type-Tr1 Tregs (FoxP3-) via plasmacytoid dendritic cell stimulation. Additionally increase in gut homing of IL-10 producing regulatory T cells and differentiation of Tregs is induced by retinoic acid generated by dendritic cells in presence of microbial antigens.

5.10 Conclusions

To date, our understanding of mechanism of FMT in modulation colonic inflammation have been limited primarily to animal models of IBD studies with human studies exploring largely changes in gut microbial profiles and peripheral immune cell changes. This study provides the most comprehensive evidence to date on changes in host biology following FMT in patients with ulcerative colitis. Pivotal findings highlighted from these analyses demonstrate that responders to FMT demonstrate a significant increase in gut homing regulatory cells, a reduction in proinflammatory Th17 cells, significant downregulation of anti-microbial defence (associated with reduction in anti-microbial peptide gene expression) and proinflammatory immunological pathways and an increase in butanoate and propanoate metabolic pathways. Assembling these findings to piece together likely biological process would help elucidate understandings of mechanisms that underlie FMT induced regulation of colonic inflammation in UC.

CHAPTER 6

Colonic mucosal regulation of bile acid signalling and mechanisms of primary sclerosing cholangitis - inflammatory bowel disease

6.1 Abstract

Background and Aims: The distinctions seen in clinical manifestations and predisposing genes for primary sclerosing cholangitis-inflammatory bowel disease (PSC-IBD) compared to ulcerative colitis (UC), support distinguishing pathobiologies. We sought to define further the pathophysiologic differences in PSC-IBD and UC, by applying a comparative and integrative systems biology approach.

Methods: Colonic biopsies were collected from patients with PSC-IBD (n=10), UC (n=10) and healthy controls (HC; n=10). Shotgun RNA-sequencing for differentially expressed genes (DEGs), immunophenotyping and 16S rRNA analysis for microbial community profiling were performed and multi-omic integration conducted.

Results: The colonic transcriptome differed significantly between groups (P=0.01). We identified 1343 DEGs and 4312 DEGs comparing HC with PSC-IBD and UC respectively, of which only 939 were shared. Pathways predominantly associated with immune response and microbial defence were upregulated in both disease cohorts. There were 1692 DEGs between PSC-IBD and UC. Pathways associated with bile acid signalling were significantly upregulated in PSC-IBD compared to UC (P=0.02). Th17 cells and IL17 producing CD4 cells were increased in both PSC-IBD and UC when compared to HC (P<0.05). Microbial profiles of the three groups were distinct (P=0.01) and associated with upregulation of bile salt transforming bacteria in PSC-IBD. Multi-omic integration revealed networks involved in bile acid homeostasis and cancer regulation as characteristic of PSC-IBD.

Conclusions: Our integrative analyses demonstrate differences in the colonic mucosal biology of patients with PSC-IBD as compared to UC. Colonic disease in PSC-IBD appears to be contributed to by mucosal bile acid toxicity, with dysregulation of bile acid signalling alongside enteric dysbiosis.

6.2 Introduction

Primary sclerosing cholangitis (PSC) is characterised by progressive inflammation and fibrotic stricturing of the biliary tree (303, 304). The pathogenesis of PSC is poorly understood and is presumed to reflect the multifactorial interplay of immune dysregulation, gut microbial dysbiosis and changes in bile acid homeostasis in a genetically at risk individuals (157, 158, 237). Due to lack of effective medical therapy it is a leading indication for liver transplantation in Western countries (305). PSC is highly comorbid with inflammatory bowel disease (IBD), which is ultimately diagnosed in approximately 75% of patients primarily in a clinical form of ulcerative colitis. PSC-IBD however behaves phenotypically differently to conventional UC (306). Patients with PSC-IBD have a milder but more extensive form of colonic inflammation (pancolitis) with rectal sparing. Although the colitis in PSC-IBD usually follows a quiescent course, patients with PSC-IBD have a significantly higher risk of developing colorectal cancer compared to UC patients without PSC (307). Large-scale genome wide association studies (GWAS) have identified multiple shared and non-shared genetic loci that underlies this risk (308-311). A substantial component of the genetic architecture of PSC-IBD is however, not shared with UC and genetic correlation modelling generates only a UC comorbidity rate of 1.6% in patients with PSC. Furthermore, microbiota-based investigations have additionally reported distinct bacterial species profiles when evaluating PSC-IBD against classical UC without liver disease. These phenotypic and genotypic differences have led to the proposal that colonic inflammation in the context of PSC-IBD is aetiologically and pathophysiologically different to that seen in UC. The colonic mucosal gene expression profile (transcriptome) in PSC-IBD, however, has not been explored before.

To appreciate further the complex and inter-dependent mechanisms underlying the colonic presentation of PSC-IBD we have undertaken a systems biology approach to highlighting

distinguishing features of PSC-IBD as compared to UC. To do so we have captured the colonic mucosal transcriptome, mucosal immunophenotype and mucosally adherent gut microbiota profiles in patients with PSC-IBD, UC alone and healthy controls. Through this approach, we model interactions between different biological systems to derive key processes that associate with distinctions in colonic phenotype seen between PSC-IBD and UC, and notably identify bile acid homeostasis in the colon as a key feature in PSC-IBD patients.

6.3 Results

Patients with PSC-IBD, UC and HC were recruited from clinic and endoscopy lists. PSC-IBD and UC were documented in keeping with European guidelines on diagnosis (EASL and ECCO). Healthy subjects had no known co-morbidities and normal colonoscopy (other than haemorrhoids) as part of investigation for rectal bleeding. Subjects were excluded if they had taken antibiotics and/or probiotics in the last three months. Only patients with large duct PSC were recruited as part of the PSC-IBD cohort and alternative aetiologies were excluded for all patients. Colonic mucosal biopsies were taken from the sigmoid colon and collected on ice in both Qiagen RNAlater tubes (for storage in -80 C) and Miltenyi Biotec gentleMACS C-Tubes containing complete RPMI media (for immediate processing). Ethical approval was given by University of Birmingham Human Biomaterials Resource Centre (HTA Licence 12358). An overview of the methodology for this study is summarised in Figure 6 - 1.

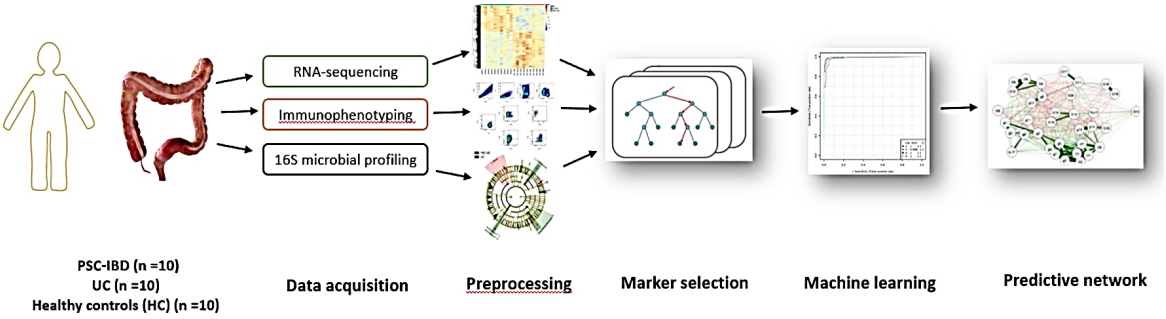


Figure 6 - 1 : Overview of data analysis.

Colonic biopsies taken from ten patients with PSC-IBD, UC and healthy controls (HC) were analysed for gene expression profiles by RNA-sequencing, immunophenotype and 16S microbial profile. Features / markers were selected based on supervised and unsupervised machine learning methods for multi-omics integration.

6.3.1 Patient demographics

Thirty patients were recruited into the study - 10 healthy controls, 10 with PSC-IBD and 10 UC. The patients between the 3 groups were matched for age and gender ratio. Patients with PSC-IBD and UC were matched for UC disease characteristics and no significant differences were identified in disease extent, Mayo endoscopic sub-score and medication use. Three patients with PSC-IBD were post-transplant and three were on ursodeoxycholic acid (UDCA). Detailed demographics are shown in Table 6 - 1.

Table 6 - 1 : Study cohort demographics

	HC	PSC-IBD	UC
Age (mean)	47.2 years	42.6 years	39.9 years
Gender M:F	6:4	7:3	7:3
Ulcerative colitis characteristics			
Pan UC		10/10 (100%)	8/10 (80%)
Mayo endoscopic subscore			
0		7/10 (70%)	8/10 (80%)
1		0/10 (0%)	0/10 (0%)
2		1/10 (20%)	2/10 (20%)
3		2/10 (20%)	0/10 (0%)
Drugs			
Mesalazine		9/10 (90%)	10/10 (100%)
Azathioprine		1/10 (10%)	2/10(2%)
Vedolizumab		1/10(10%)	0/10(0%)
Other biologics		0/10 (0%)	0/10(0%)
Serum liver tests (median [IQR])			
Albumin (g/L)		48 [5]	46 [2.2]
Bilirubin (µmol/l)		22.5 [24]	13.5 [3.5]
ALT (IU/l)		45.1 [17]	26.1 [5.6]
ALP (IU/l)		181 [178]	74.8 [15.3]
Liver disease characteristics			
Cirrhosis		1/10 (10%) *	
On UDCA		3/10 (30%)	
Post OLT		3/10 (30%)	
Anti-rejection drugs			
Tacrolimus		3/3	
Mycophenolate		1/3	

6.3.2 Colonic mucosal transcriptome

Quality control

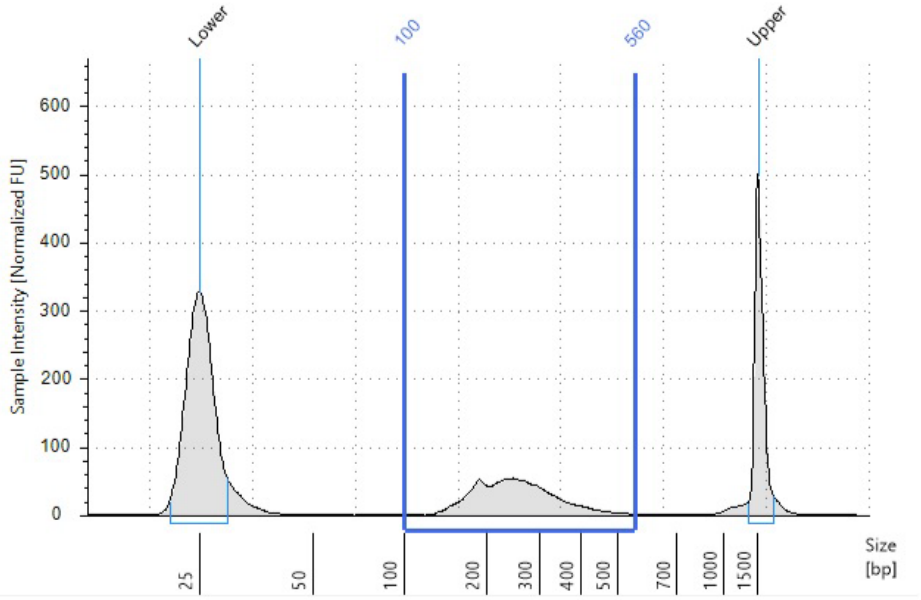


Figure 6 - 2 : RIN and library QC

RIN of at least 8 was maintained through the library prep for this study as shown in the final pooled library QC check

An average of 38 million 75bp PE reads (+/- 8.3 million) were generated per sample. Following quality control trimming and in silico decontamination of ribosomal RNA reads, an average of 35 million paired end reads were then processed for mapping and subsequent differential gene expression analysis. Library QC is shown in Figure 6 - 2 and sequencing QC is shown in Figure 6 – 3.

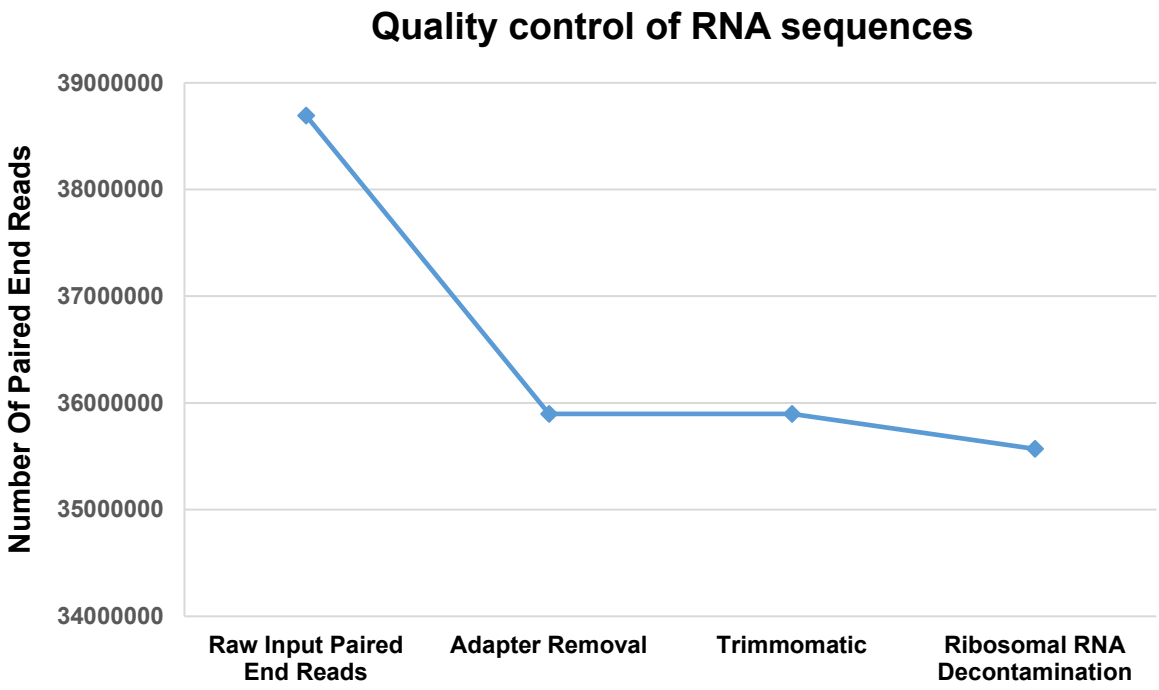


Figure 6 - 3 : Quality control of RNA sequences

Graphical representation of QC of RNA sequences. An average of 8% of paired end sequences were discarded from input raw sequences following adapter removal and trimming based on Phred score and minimum length (as described in methods). A further average of 1% of reads were removed following insilico ribosomal RNA decontamination.

6.3.3 Effect of colonic segment biopsy site and stability of RNA expression

RNA expression was similar in the ascending, descending and transverse colon based on for transcriptomic analysis of a healthy control as shown in Figure 6 - 4. The RNA expression was also similar in three biopsies taken from the same site. PCA plot demonstrated that RNA expression from colonic biopsies taken from the same healthy individual clustered together and away from other cohorts. This suggested that there is no (or minimal) intra-individual variability in RNA expression in healthy controls.

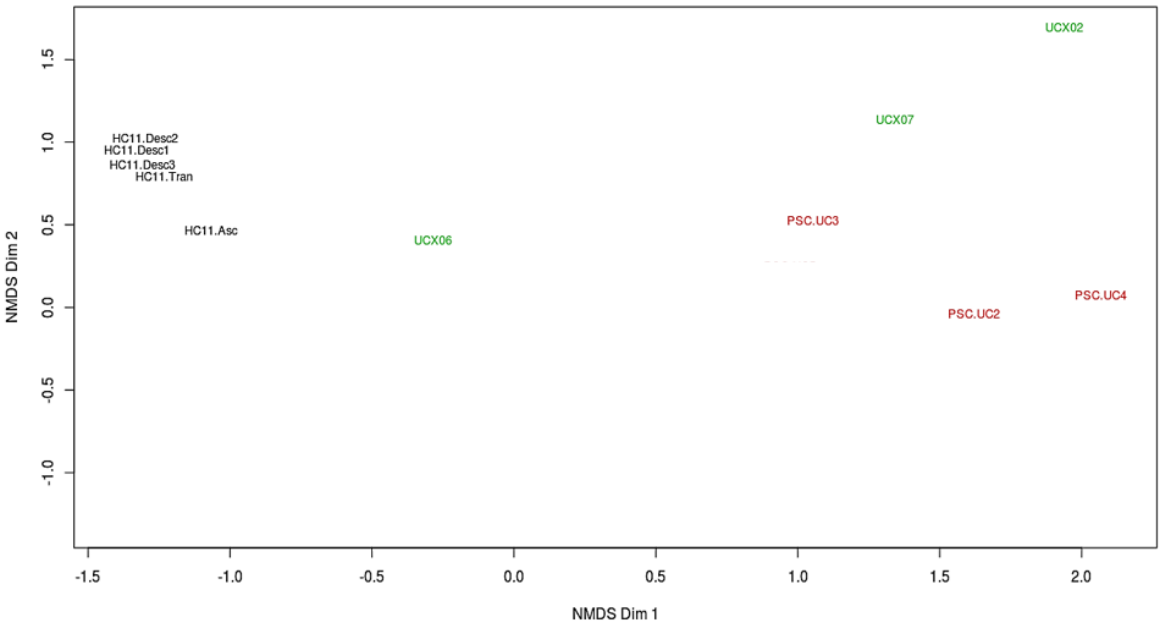


Figure 6 - 4 : Intra-individual stability of RNA expression (for healthy control)

PCA plot demonstrating RNA expression from colonic biopsies taken from the same healthy individual clustered together and away from other cohorts. Black font represents different biopsy sites healthy controls.

6.3.4 The mucosal transcriptomic landscape is significantly different between PSC-IBD, UC and healthy controls

The mucosal transcriptomic profile differed significantly between the three cohorts (p=0.01; Monte Carlo simulation). Principal Component Analysis (PCA) to study the three way and two way variations between the groups showed segregation of the cohorts (Figure 6 - 5, 6-6). The highest variation was observed between PSC-IBD and HC (PC1 - 59.7%) whereas the lowest variation was observed between PSC-IBD and UC (PC1 - 42%).

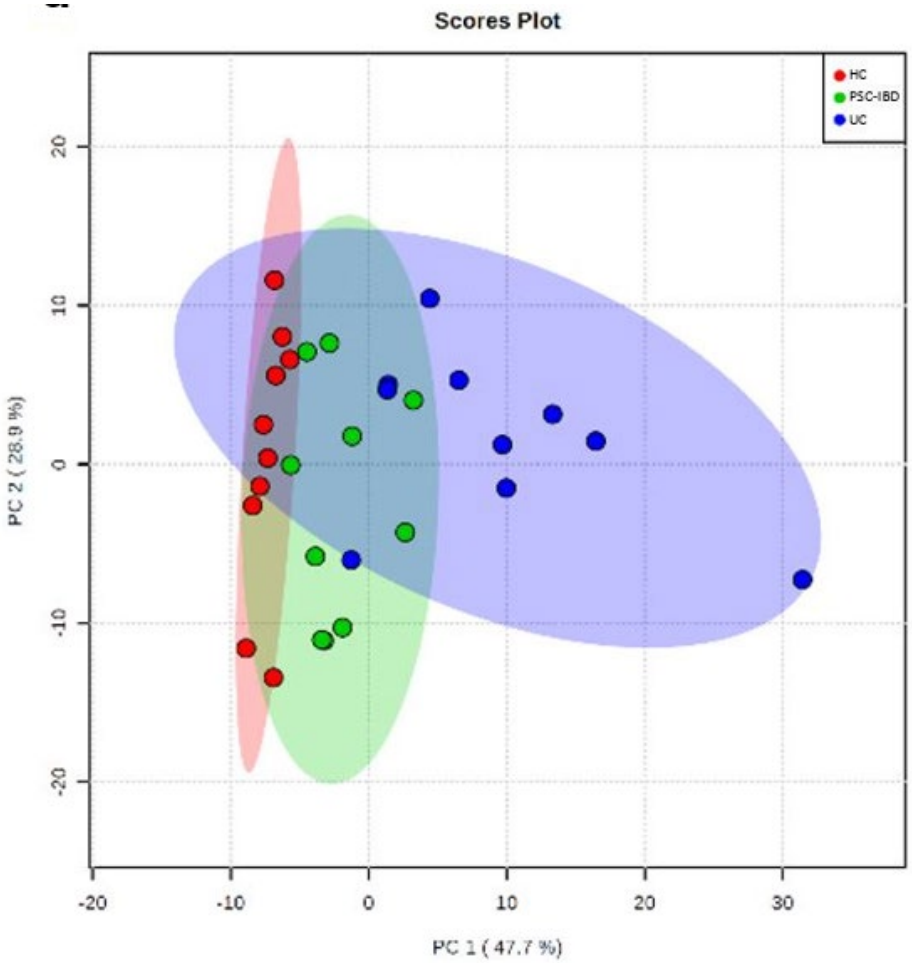


Figure 6 - 5 : Principal component analysis (PCA) score plot performed on the mucosal transcriptome datasets between all cohorts.

Demonstrates clustering of subjects within, and variation between cohorts. Dots represents samples and are coloured according to the subject cohort. Ellipse represents 95% confidence. Results are plotted according to the PC1 and PC2 scores with the percent variation explained by the respective axis. PCA plots demonstrating variation between HC, UC and PSC-UC samples. The three groups were significantly different from each other (p = 0.01).

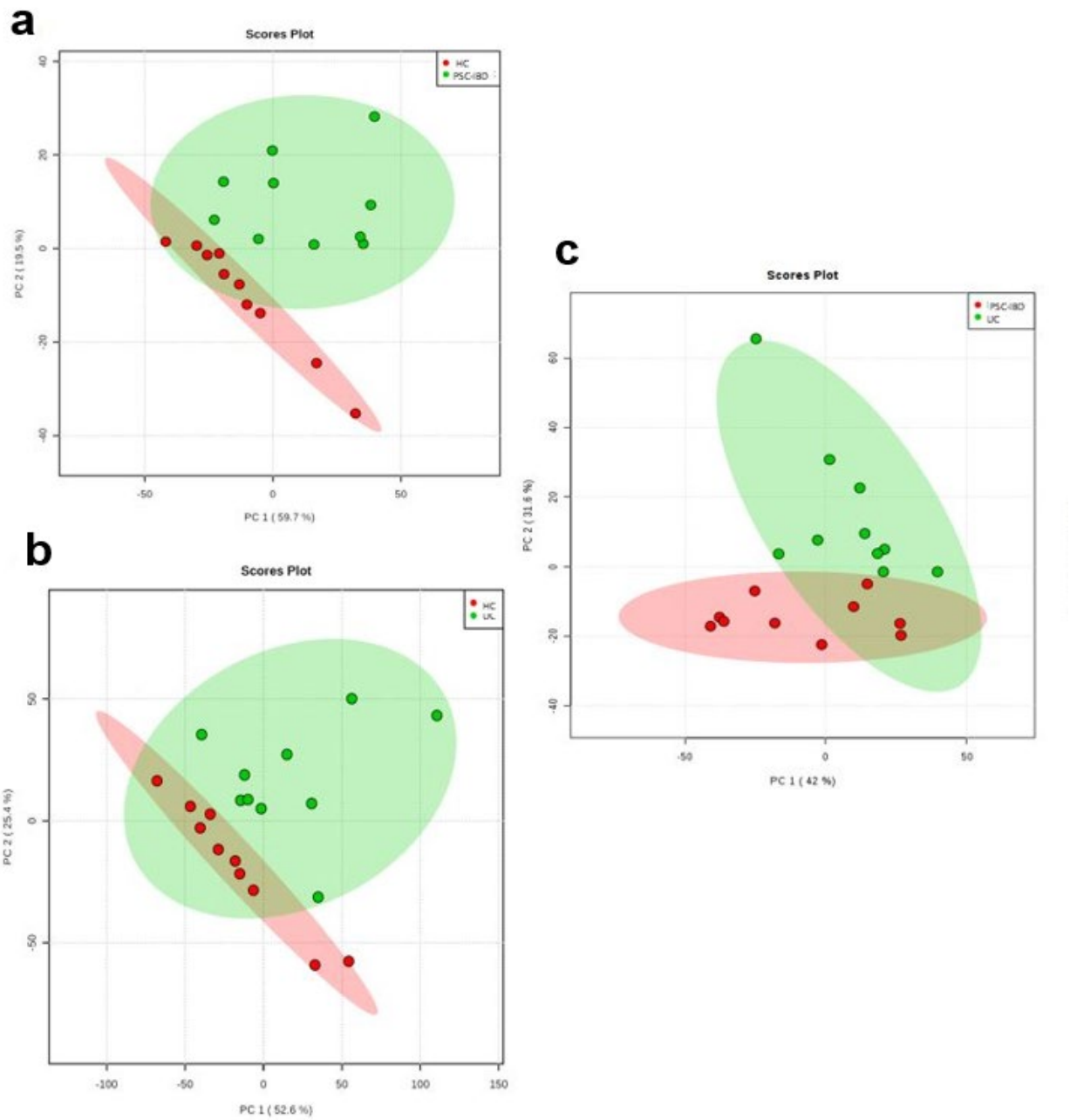
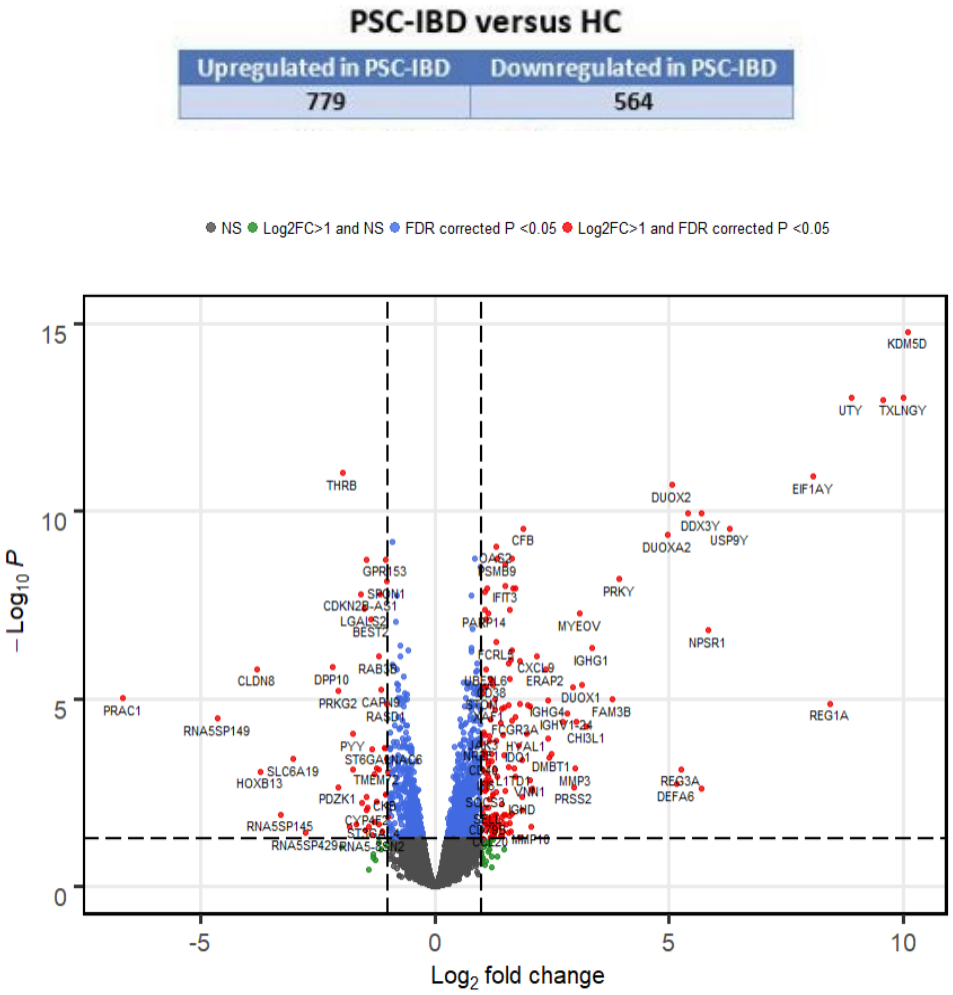


Figure 6 - 6 : Principal component analysis (PCA) score plot performed on the mucosal transcriptome datasets between each cohort.

Dots represents samples and are coloured according to the subject cohort. Ellipse represents 95% confidence. Results are plotted according to the PC1 and PC2 scores with the percent variation explained by the respective axis. **(a)** PSC-IBD versus HC; **(b)** UC versus HC; **(c)** PSC-UC versus UC

PSC-IBD vs HC

In comparison to healthy controls we found 1343 genes in PSC-IBD were differentially expressed (of which 779 were upregulated and 564 were downregulated) and 4312 genes in UC were differentially expressed (of which 2189 were upregulated and 2123 were downregulated) (Figure 6 - 7).



Upregulated	
Gene name	logFC
KDM5D	10.06545
TXLNGY	9.922408
RPS4Y1	9.527807
UTY	8.865803
REG1A	8.414581
EIF1AY	8.050854
USP9Y	6.269043
DEFA5	5.729388
NPSR1	5.683884
DDX3Y	5.64706

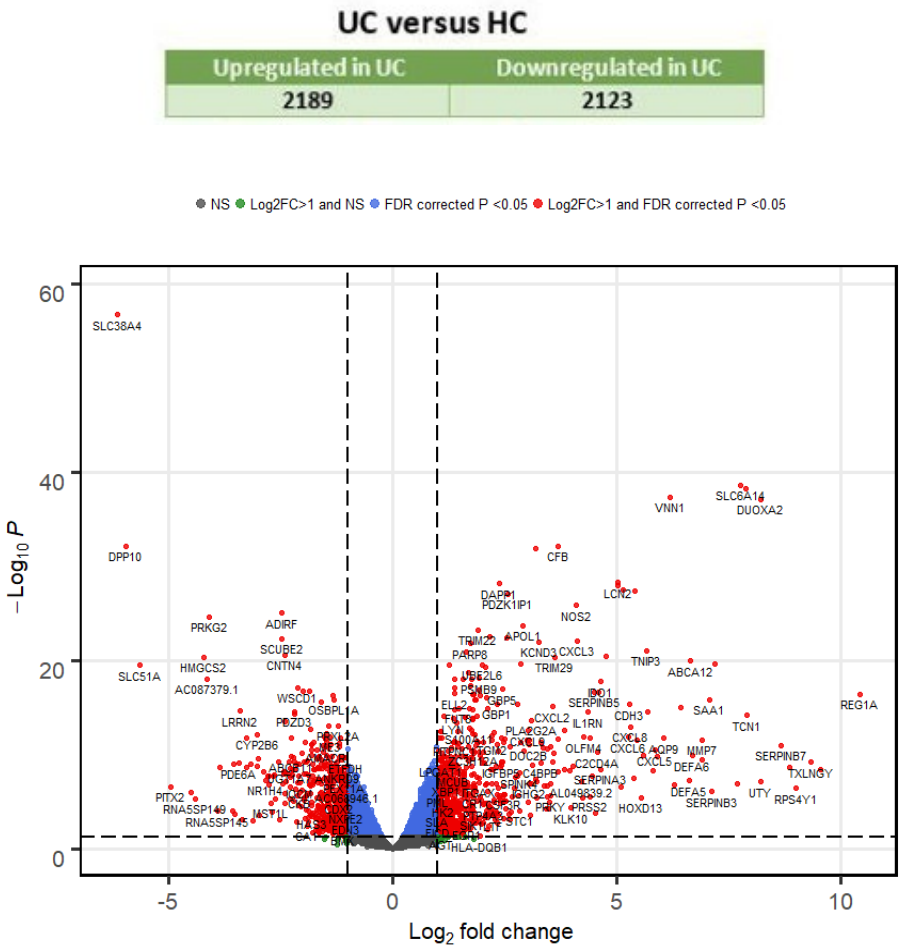
Downregulated	
Gene name	logFC
PRAC1	-6.60074
RNA5SP149	-4.54462
CLDN8	-3.76302
HOXB13	-3.73685
RNA5SP145	-3.13464
SLC6A19	-3.0583
DPP10	-2.21029
PDZK1	-2.07906
PRKG2	-2.05916
THRB	-1.9656

Figure 6 - 7 : Differential gene expression profiles and volcano plots between PSC-IBD and HC

Volcano plots demonstrating log2 fold change of genes against negative log10 P value. Top 10 upregulated and downregulated differentially expressed genes listed in table. Full list available as supplementary table.

UC vs HC

In comparison to healthy controls, 4312 genes in UC were differentially expressed (of which 2189 were upregulated in UC and 2123 were downregulated) as shown in Figure 6 - 8). Upregulated genes were associated with multiple pathways linked to immune activation.



Upregulated	
Gene name	logFC
REG1A	10.40383
KDM5D	9.51123
TXLNGY	9.310356
RPS4Y1	8.953721
REG1B	8.799701
SERPINB7	8.725266
DUOXA2	8.239814
UTY	8.193202
TCN1	7.922242
DUOX2	7.819073

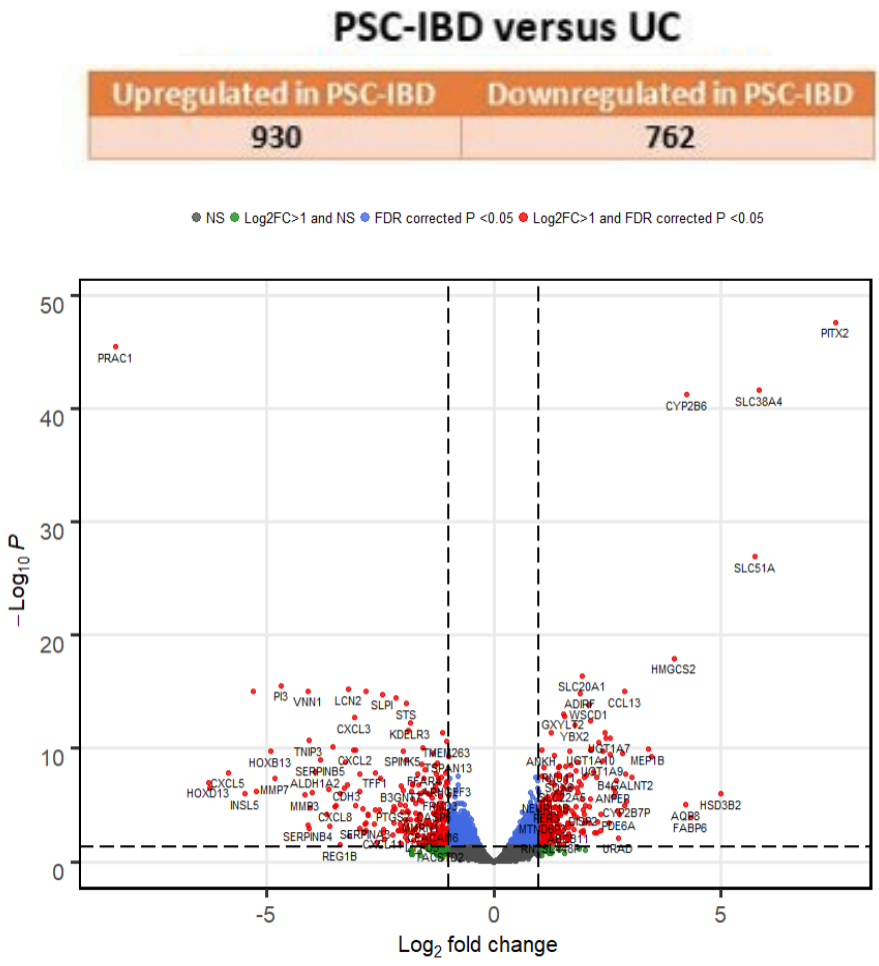
Downregulated	
Gene name	logFC
SLC38A4	-6.19254
DPP10	-5.85008
SLC51A	-5.61959
PITX2	-5.09375
AQP8	-4.44459
RNA5SP149	-4.37537
HMGCS2	-4.17666
PRKG2	-4.07295
AC087379.1	-4.05714
RNA5SP145	-3.89751

Figure 6 - 8 : Differential gene expression profiles and volcano plots between UC and HC

Volcano plots demonstrating log2 fold change of genes against negative log10 P value. Top 10 upregulated and downregulated differentially expressed genes listed in table. Full list available as supplementary table.

PSC-IBD vs UC

Only 939 genes had shared differential expression in PSC-IBD and UC when compared to healthy controls (of which 588 were upregulated and 351 were downregulated). On comparing PSC-IBD directly to UC 1692 genes were differentially expressed (of which 930 were upregulated and 732 downregulated), (Figure 6 - 9, 6 - 10). The full list of differentially expressed genes with pairwise comparisons of the three groups is available as Appendices 1.



Upregulated	
Gene name	logFC
PITX2	7.515611
SLC38A4	5.851151
SLC51A	5.715371
HSD3B2	5.005883
FABP6	4.257956
CYP2B6	4.243903
AQP8	4.171683
HMGCS2	3.939901
AC087379.1	3.40867
MEP1B	3.383144

Downregulated	
Gene name	logFC
PRAC1	-8.28923
HOXD13	-6.33457
SERPINB7	-6.26739
CXCL5	-5.95677
INSL5	-5.48694
SLC6A14	-5.23739
AC108865.1	-5.23026
HOXB13	-4.89692
MMP7	-4.81336
PI3	-4.69795

Figure 6 - 9 : Differential gene expression profiles and volcano plots between PSC-IBD and UC

Volcano plots demonstrating log2 fold change of genes against negative log10 P value. Top 10 upregulated and downregulated differentially expressed genes listed in table. Full list available as supplementary table.

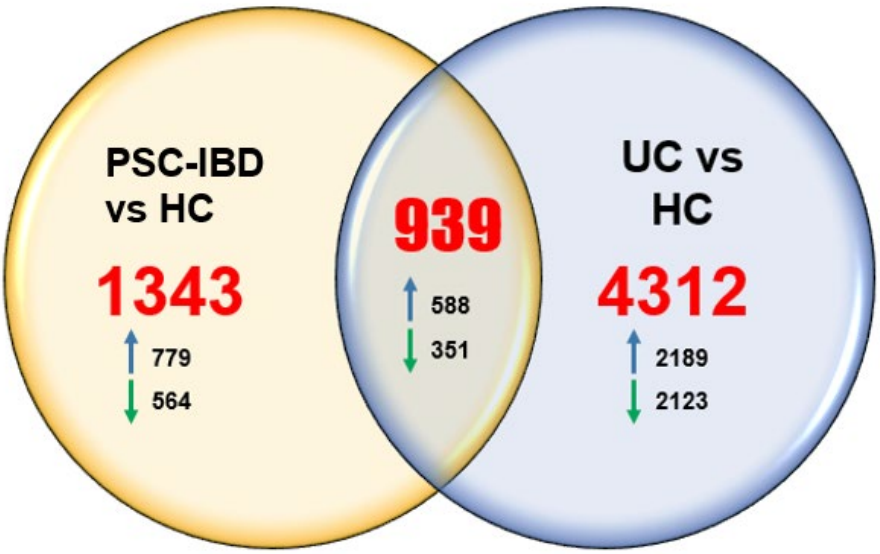


Figure 6 - 10 : Venn diagram showing shared DEG in PSC-UC and UC when compared to HC.

Only 939 genes had shared differential expression in PSC-IBD and UC when compared to healthy controls (of which 588 were upregulated and 351 were downregulated).

6.3.5 Pathway analysis

We performed pathway analysis through competitive gene set testing to assess for enrichment of a particular gene annotation category amongst the differential expression datasets. A full list of Gene Ontology and KEGG/Reactome pathways comparing the three groups is available as Appendix

PSC-IBD compared to HC

Gene ontology analysis revealed enrichment of 948 biological processes in PSC-IBD compared to HC. Of these 824 were upregulated and were primarily associated with innate, adaptive and humoral immune response (Figure 6 - 11). Furthermore, there was upregulation of processes associated with anti-microbial defence and extracellular matrix remodelling. Processes associated with specific metabolic functions and cellular proliferation were significantly downregulated. Similarly, KEGG and Reactome analysis revealed that 186 pathways that were differentially regulated in PSC-IBD compared to HC of which 130 were upregulated and associated with immunological mechanisms and signalling processes (Figure 6 - 12).

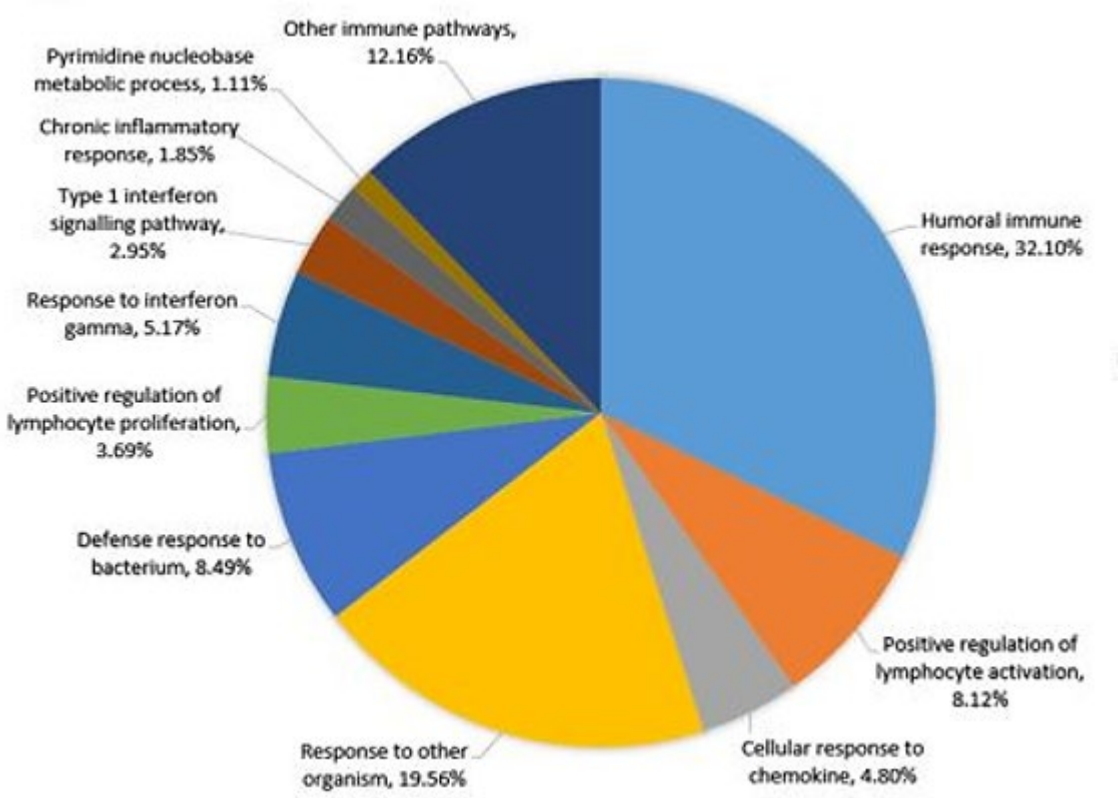
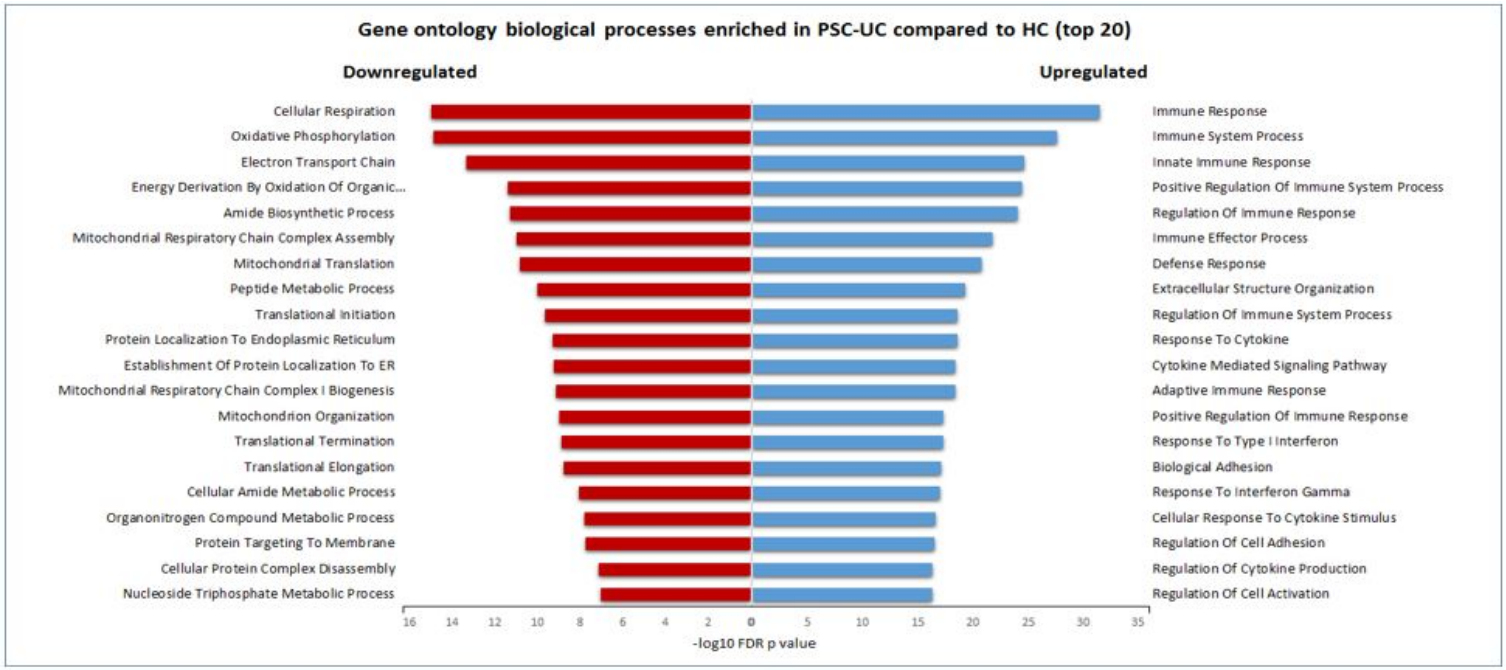


Figure 6 - 11 : Functional annotation enrichment analyses using ClueGO in PSC-IBD compared to HC

Enrichment analyses using ClueGO of upregulated genes using predefined gene ontology biological processes (log2FC >1, FDR P value < 0.05).



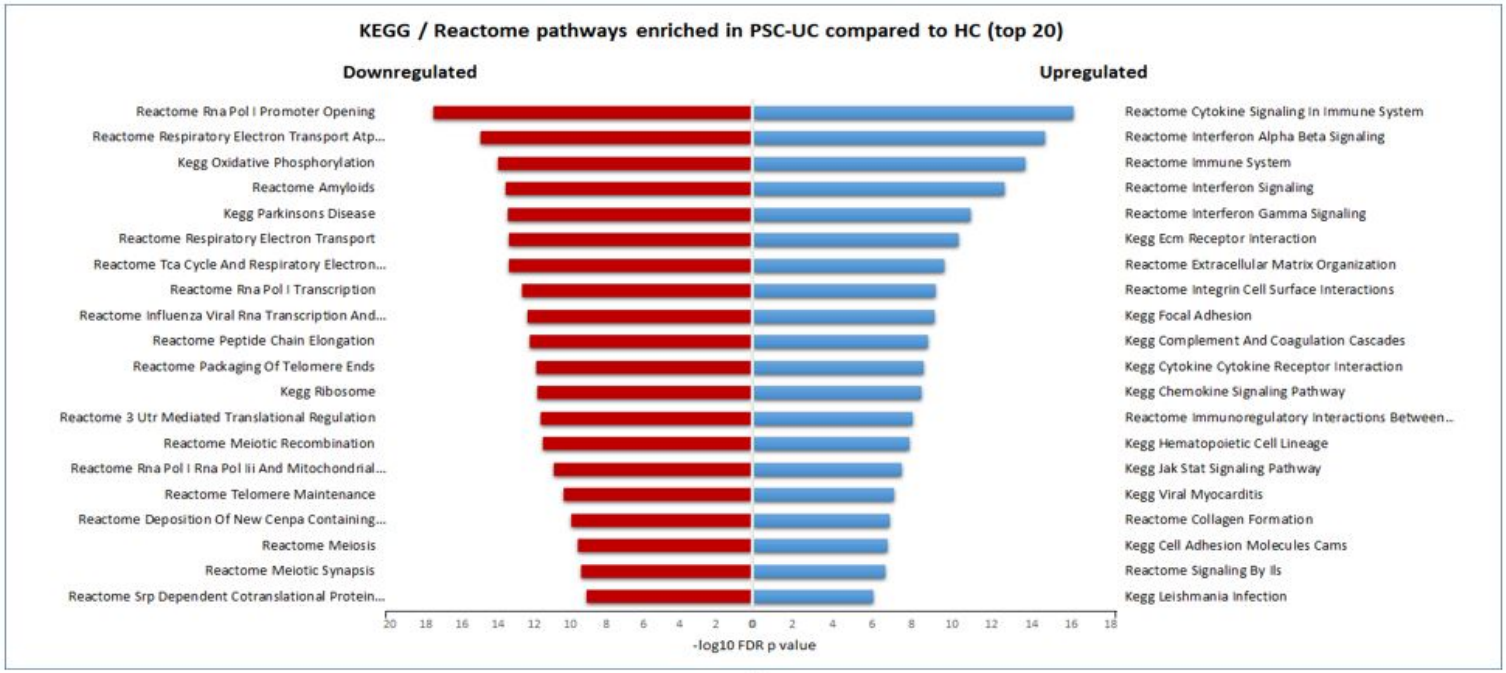


Figure 6 - 12 : KEGG/Reactome pathways in PSC-IBD compared to HC.

Top 20 gene ontology biological processes and KEGG/Reactome pathways by competitive gene set testing using Camera demonstrate upregulation of immune mediated pathways in PSC-IBD compared to HC.

UC compared to HC

Pathway analysis of UC compared to HC demonstrated that 982 gene ontology biological processes were significantly enriched of which 837 were upregulated. KEGG and Reactome analysis showed that 253 pathways were significantly enriched of which 186 were upregulated. Similar to the pathway comparison between PSC-IBD and HC, differentially upregulated biological process and pathways in these analyses were associated with a multitude of facets of immunological response, antimicrobial defence mechanisms, extracellular matrix remodelling (Figure 6 - 13). The pathways that were downregulated were associated with metabolic processes, cellular respiration, butyrate and fatty acid metabolism. (Figure 6 - 14).

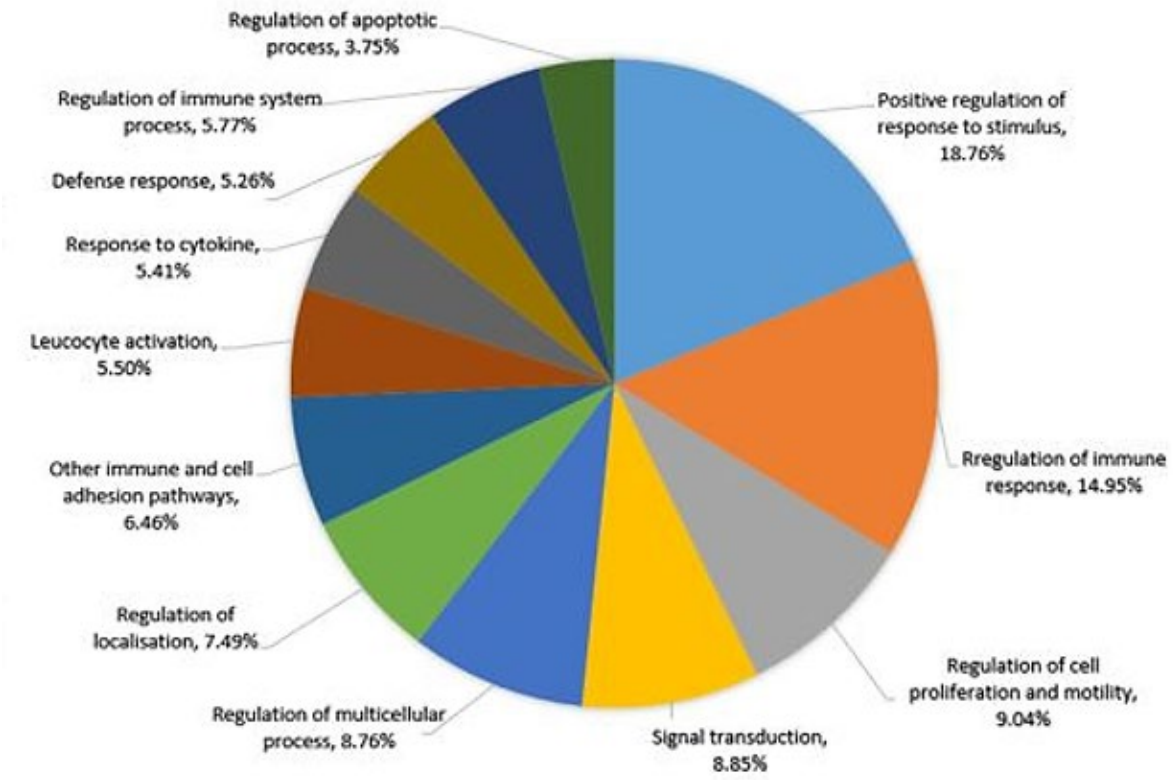
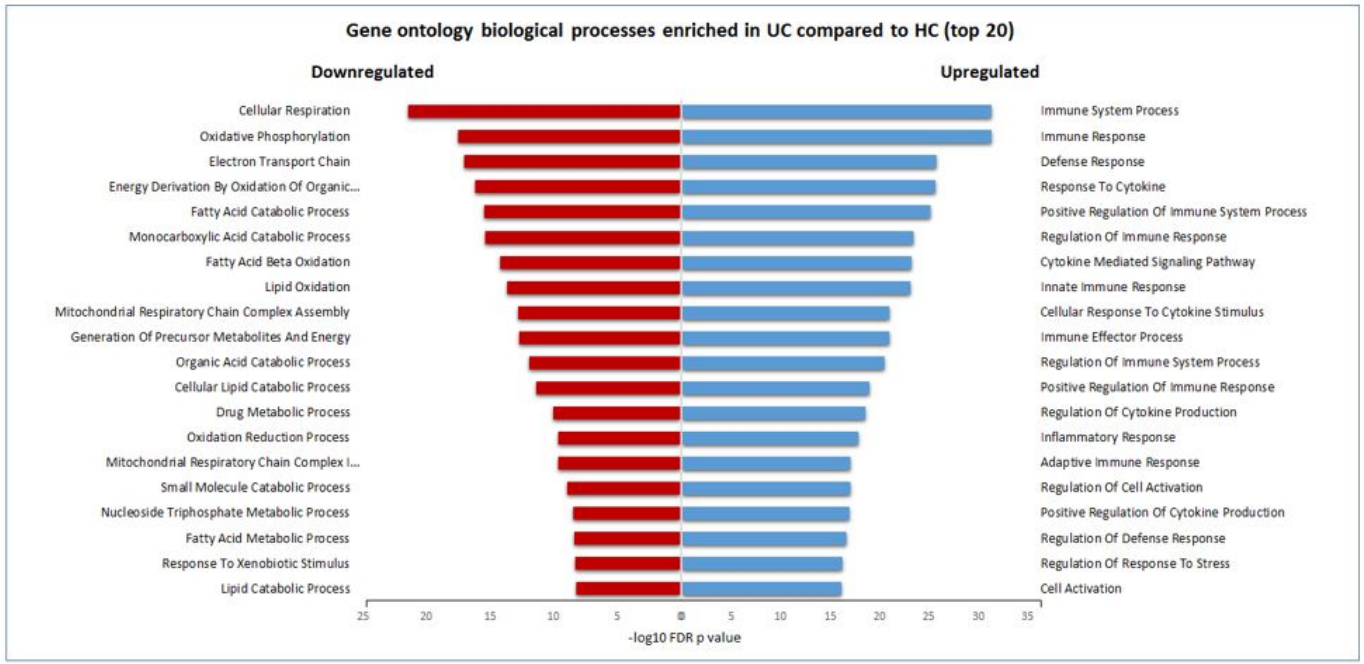


Figure 6 - 13 : Functional annotation enrichment analyses using ClueGO in UC compared to HC

Enrichment analyses using ClueGO of upregulated genes using predefined gene ontology biological processes (log2FC >1, FDR P value < 0.05).



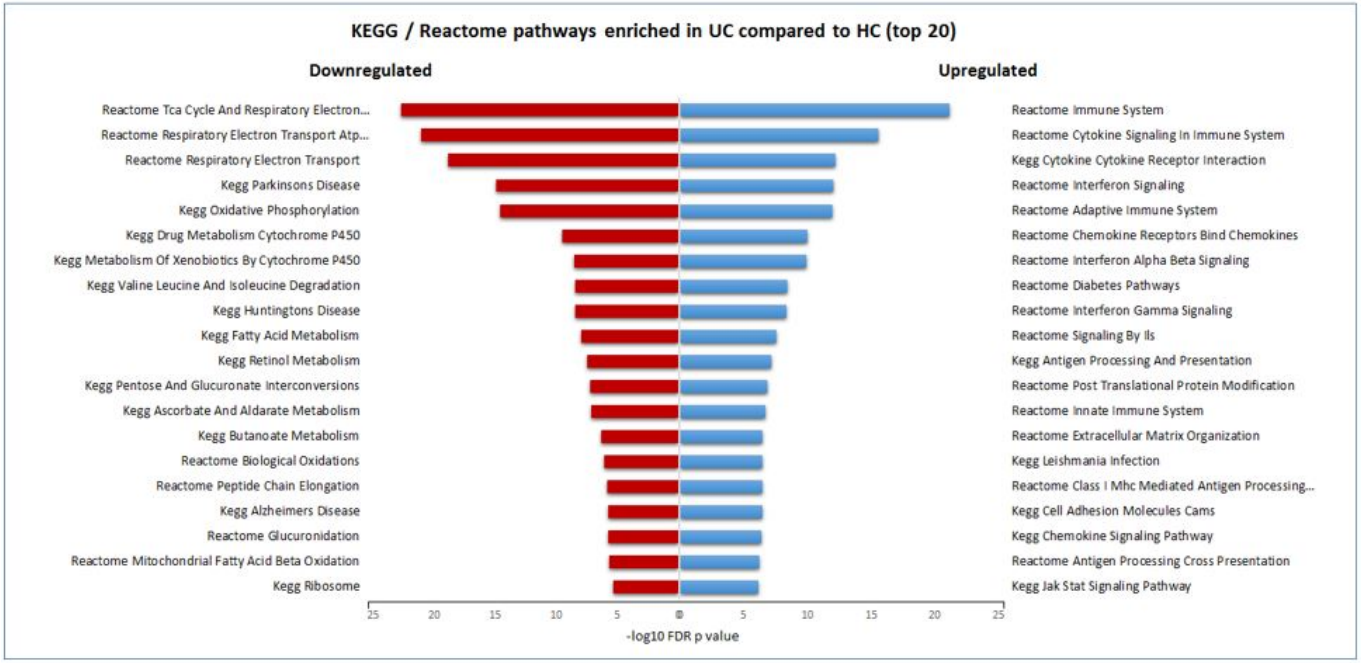


Figure 6 - 14 : KEGG/Reactome pathways in UC compared to HC.

Top 20 gene ontology biological processes and KEGG/Reactome pathways by competitive gene set testing using Camera demonstrate upregulation of immune mediated pathways in UC compared to HC.

PSC-IBD compared to UC

Gene ontology analysis comparing PSC-IBD with UC demonstrated enrichment of 563 biological processes. Of these 104 were upregulated in PSC-IBD and were associated with fatty acid metabolic processes, glucuronidation, bile acid and bile salt metabolism processes and transport. Processes such as those associated with immunological response, defence mechanisms and cell cycle were significantly downregulated. KEGG and Reactome analysis revealed that 238 pathways that were differentially regulated in PSC-IBD compared to UC of which 62 were upregulated. Findings similar to those from gene ontology enrichment analysis were demonstrated in this pathway analysis. However, additionally pathways associated with bile acid homeostasis including PPAR signalling, nuclear receptor transcription pathways, recycling of bile acids were upregulated along with butanoate and propionate metabolism in PSC-IBD compared to UC (Figure 6 - 15, 6 - 16). Pathways associated with DNA damage response, telomere, processes associated with transition of cell cycle phases and cell replication with significantly downregulated in PSC-IBD compared to UC.

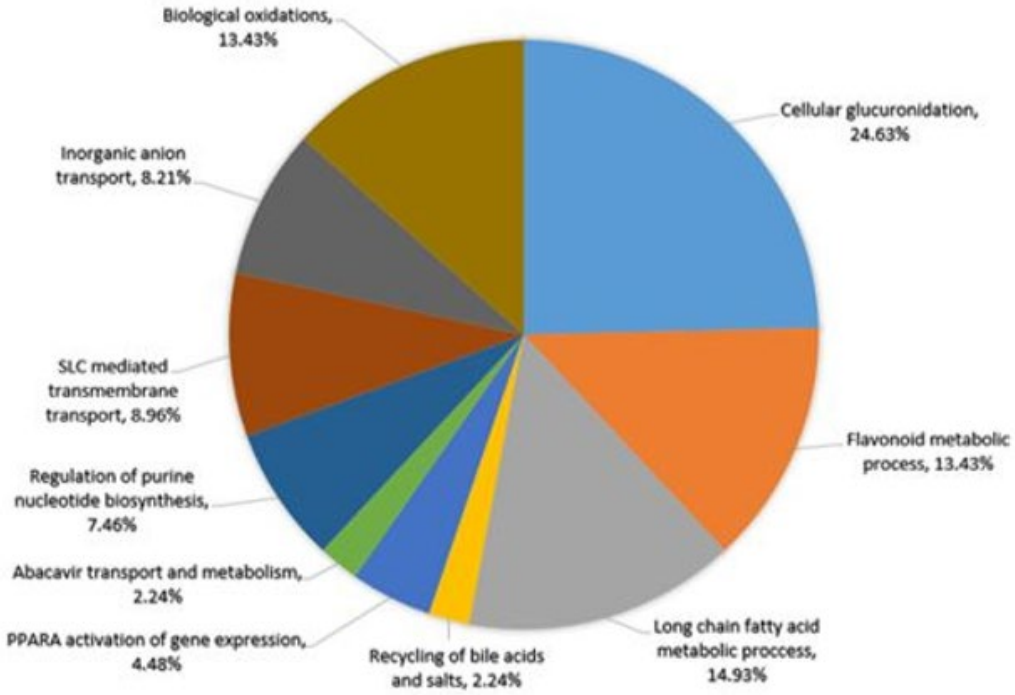


Figure 6 - 15 : Functional annotation enrichment analyses using ClueGO in PSC-IBD compared to UC

Enrichment analyses using ClueGO of upregulated genes using predefined gene ontology biological processes (log2FC >1, FDR P value < 0.05).

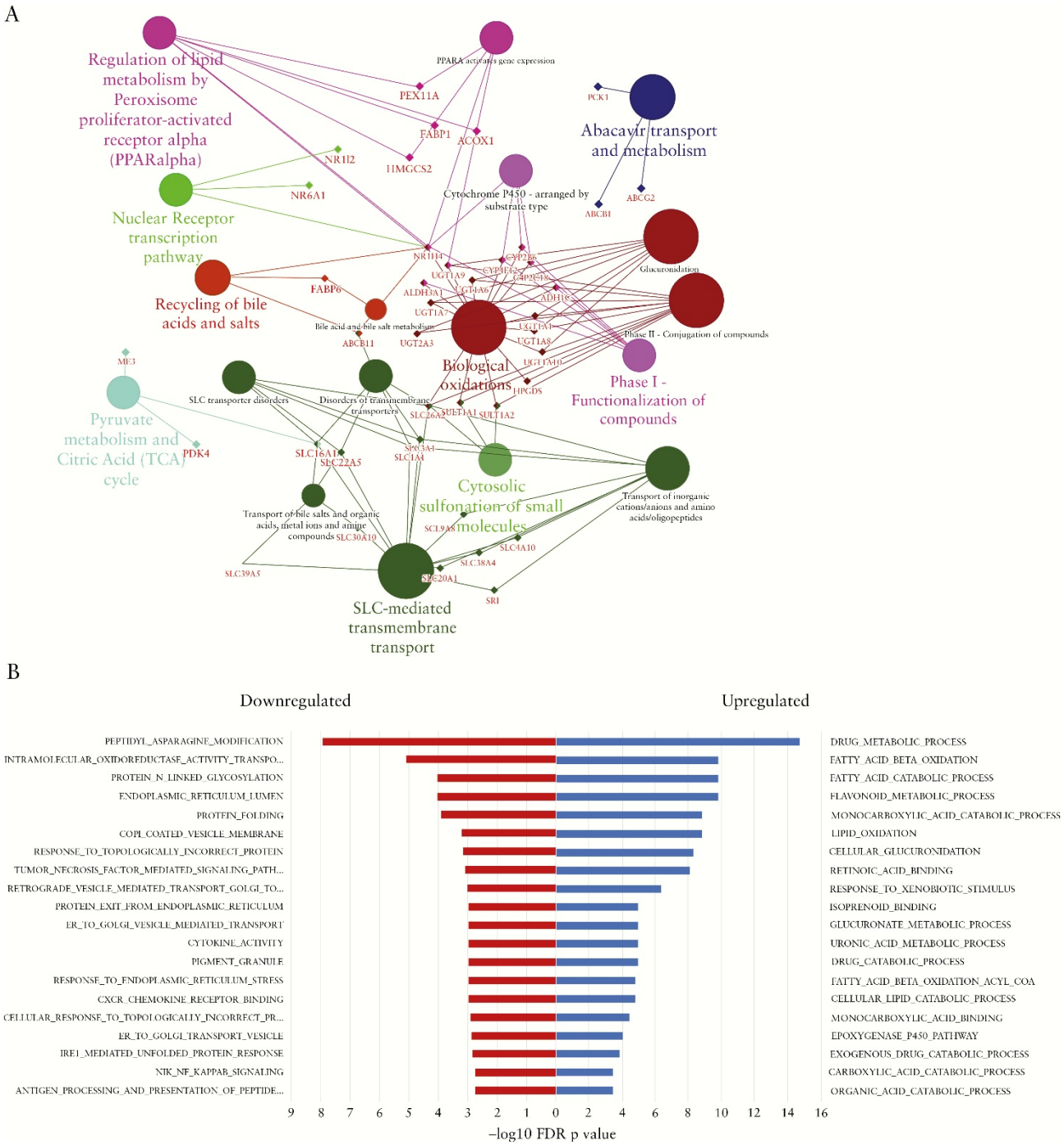


Figure 6 - 16 : Bioinformatic analysis identifies pathways including bile acid signalling as relevant to disease distinctions

(a) Detailed enrichment analysis of gene clusters in functionally grouped network. Gene networks and pathways involving key nuclear receptors involved in bile acid homeostasis such as FXR, PPAR, conjugation, binding and transport are significantly upregulated in PSC-IBD compared to controls; (b) Top 20 gene ontology biological processes PSC-IBD vs UC demonstrate metabolic pathways, many of which are involved in bile acid homeostasis are upregulated in PSC-IBD compared to UC whereas immune activation and defence pathways are upregulated in UC compared to PSC-IBD.

6.3.6 Cell deconvolution

Computational cell deconvolution showed that there were no differences in proportions of epithelial cells, CD4 and CD8 T cells, monocytes and B cells between the three groups Figure 6 - 17). There was however an increase in the dendritic cell subset in PSC-IBD compared to HC (p=0.03, mean delta= 0.004).

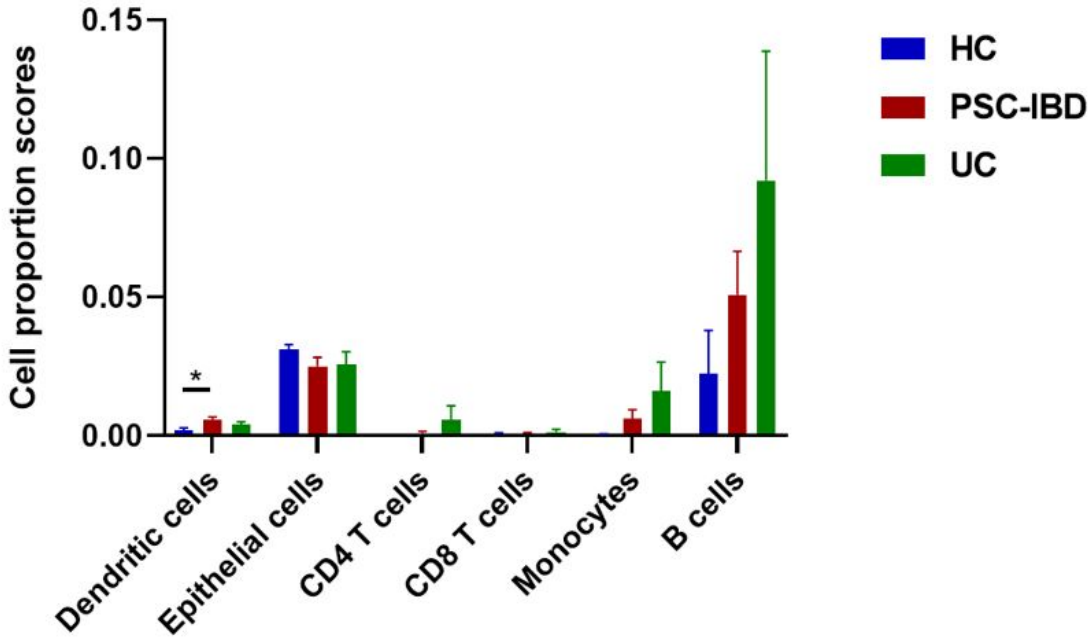


Figure 6 - 17 : Computational cell deconvolution

Computational cell deconvolution was performed to estimate the relative composition of immune cell subsets and epithelial cells in each sample. Cell subset proportion scores are generated based on the xCell pipeline's gene signature-based method. This demonstrated only an increase in dendritic cell population in PSC-IBD compared to controls (P=0.03).

6.3.7 Mucosal immunophenotyping

PSC-IBD and UC were characterised by a significantly higher frequency of colonic mucosal CCR6+CD161+ Th17 cells compared to controls (18.62% vs 8.41%; p<0.001 and 15.61% vs 8.41%; p=0.04 respectively). CCR6-CXCR3+CCR5+ Th1 cells were significantly lower in PSC-IBD compared to HC (15.38% vs 23.62% respectively; P<0.01) (Figure 6 – 18). No differences in Th1 populations were seen between UC and HC. Significantly increased frequencies of IL17 producing CD4 T cells were observed in both PSC-IBD and UC compared to HC (8.48% vs 5.67%; P<0.01 and 8.8% vs 5.67%; P=0.03 respectively). There were no differences identified in Th2, Tregs, TNF-alpha and IFN-gamma producing CD4 cells between the disease cohorts and healthy controls. There were no significant differences in any of the immunological subsets between PSC-IBD and UC.

There were no differences identified in Th1, Th2, Tregs, TNFa and IFNy producing CD4 cells between PSC-IBD and HC or UC and HC. Additionally, there were no significant differences in any of the immunological subsets between PSC-IBD and UC.

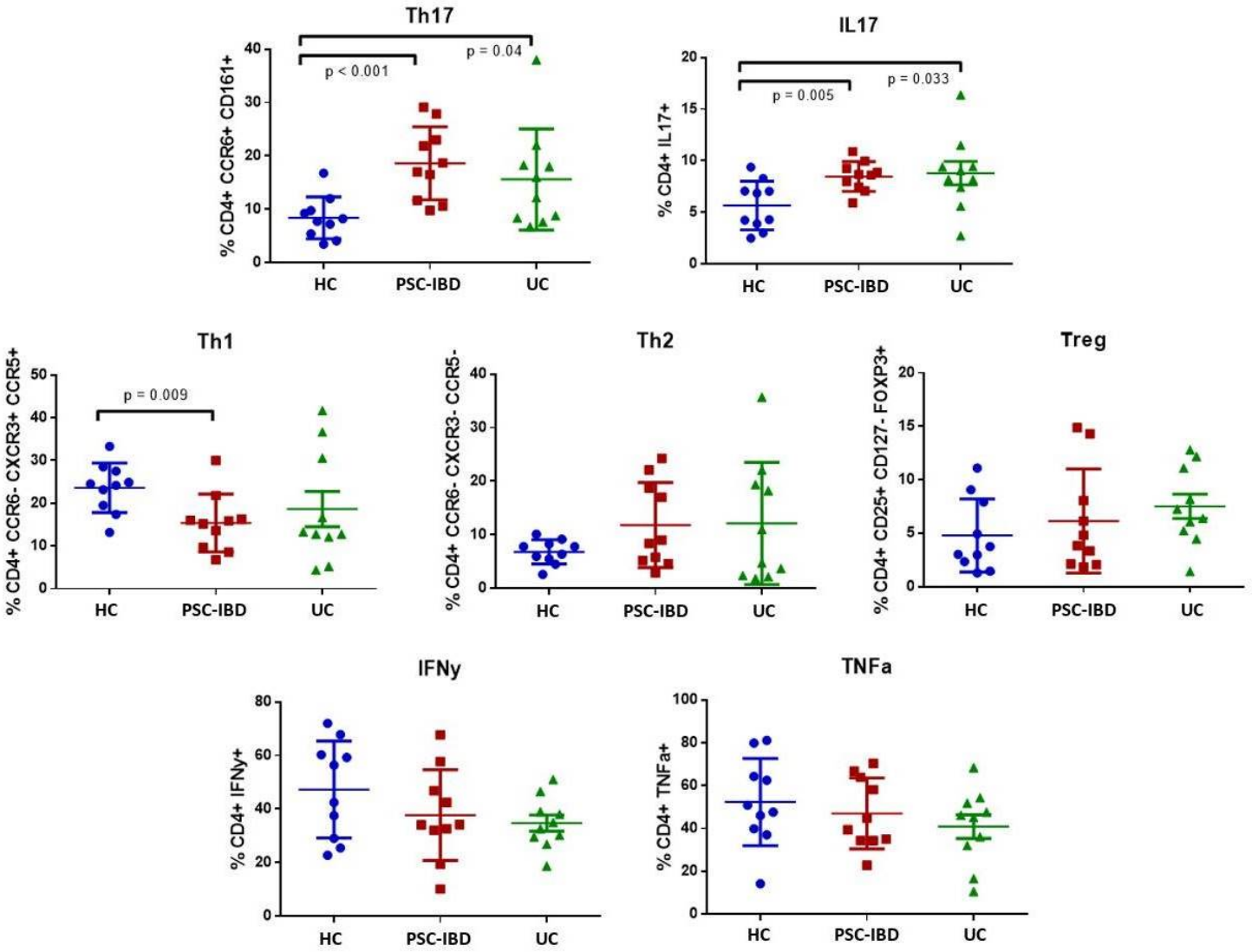


Figure 6 - 18 : Characterisation of paired mucosal immune subsets in PSC-IBD, UC and HC.

Bars denote standard error of mean. Probability values (P values) provided for significant subset comparisons. Both Th17 cells (CD4+CCR6+CD161+) and IL17 producing CD4 cells (CD4+IL17+) were significantly increased in PSC-UC and UC compared to healthy controlled. Additionally Th1 cells (CD4+CCR6-CD161-CCR5+CXCR3+) were decreased in PSC-IBD only compared to healthy controls. No changes in were identified in regulatory T cell population (CD4+CD127-CD25+FoxP3+) or in Th2 like CD4 subsets (CD4+CCR6-CD161-CCR5-CXCR3-) in either PSC-IBD or UC compared to controls. There were no differences identified between PSC-IBD and UC in any of the subsets.

6.3.8 16S rRNA sequencing

Quality control of 16S rRNA microbiota profiling

After sequencing and quality filtering (trimming and denoising), more than 6.6million reads and 3396 features were obtained. These corresponded to a mean of 110,035 reads per sample. The mean sequence length was 251 bases (range 232 – 413). There was no significant difference in the number of amplicon sequence variants (ASVs) between the three cohorts.

Minimum:	8920
Median:	179726.0
Mean:	180759.564516
Maximum:	761054
Total:	11207093

Figure 6 - 19 : Demultiplexed sequence counts summary

A total of 11,207,093 sequences were recovered with a median sequencing depth of 180,759 per sample.

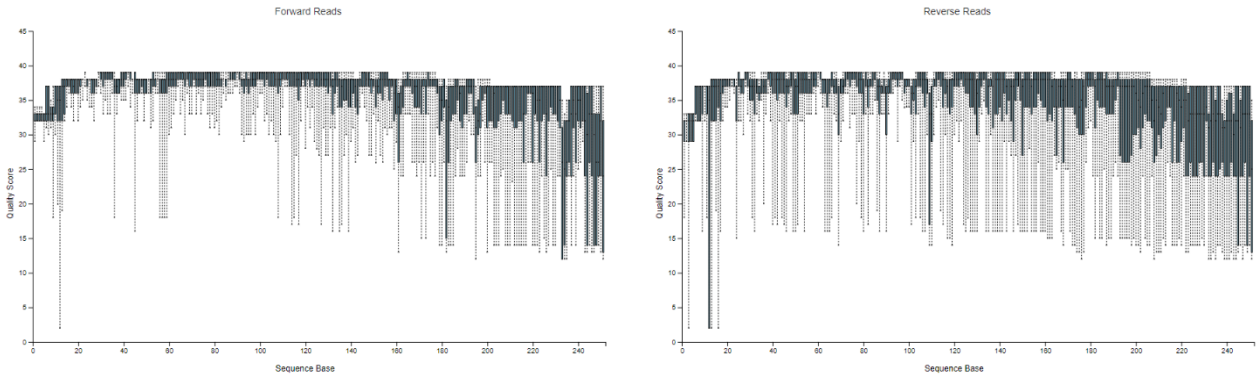


Figure 6 - 20 : QC plot for filtering and trimming of reads

Visualisation of QC plot generated using a random sampling of 10000 out of 4028486 sequences without replacement. Y-axis denotes Phred quality scores (Q scores) and X-axis denotes sequence base number. Forward reads were trimmed at position 43 and truncated to position 232. Reverse reads were trimmed at position 13 and truncated to position 243. As V4 region of the 16S gene is 254bp there would be sufficient overlap in forward and reverse reads for alignment.

Alpha diversity indices

No significant differences in alpha diversity (richness and evenness) were observed between the three groups as shown in Figure 6 – 21.

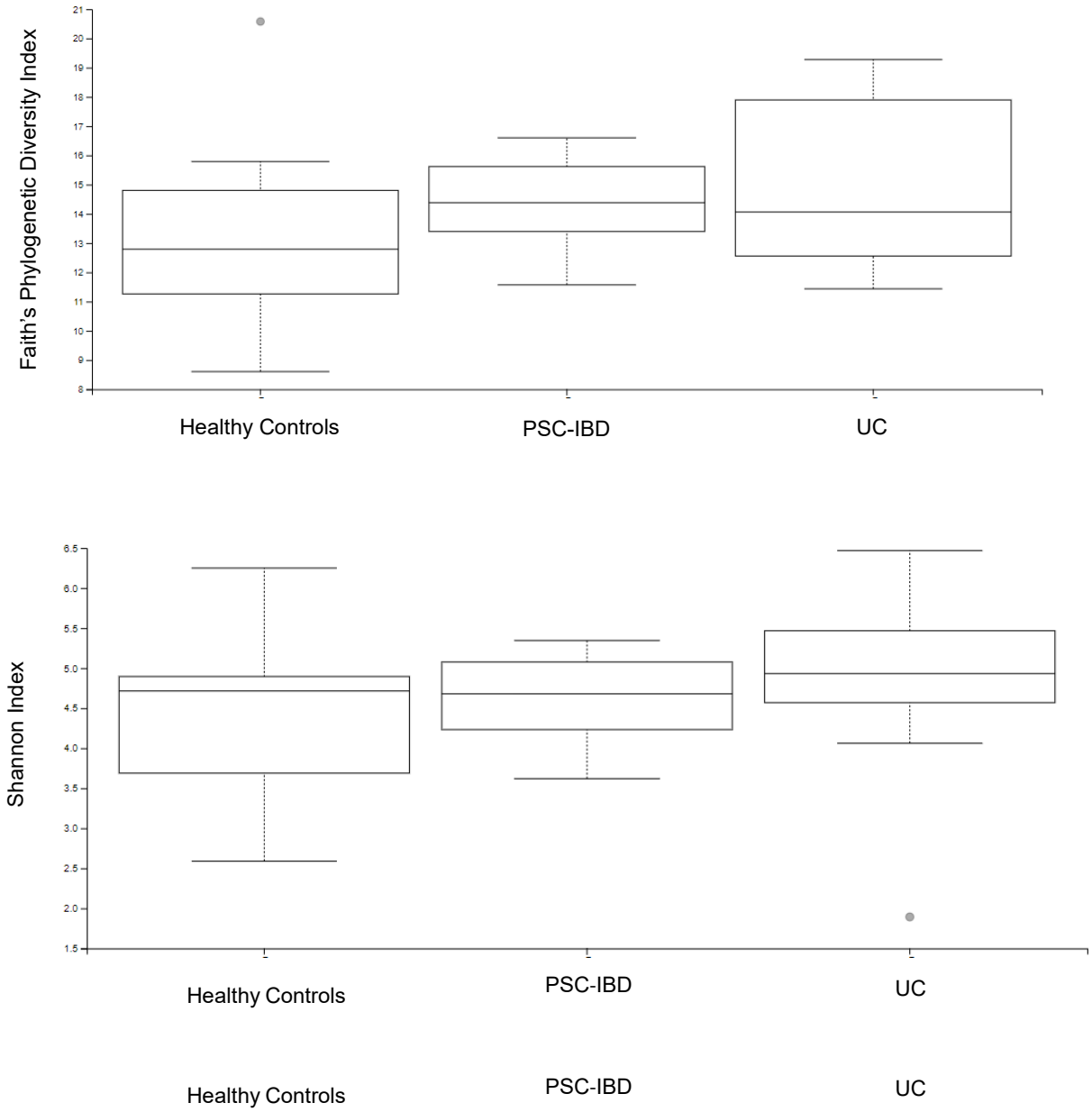


Figure 6 - 21 : Alpha diversity analysis comparing cohorts

As estimated by Shannon and Faith's diversity index analysis demonstrated no significant difference in richness and diversity between the three cohorts.

Beta diversity indices

Gut microbial profiles differ significantly between the three groups

The microbial profiles of the three groups were significantly different to each other based on their beta-diversity (p=0.01). Additionally, the colitis phenotype (PSC-IBD and UC) was significantly different to HC (p=0.007).

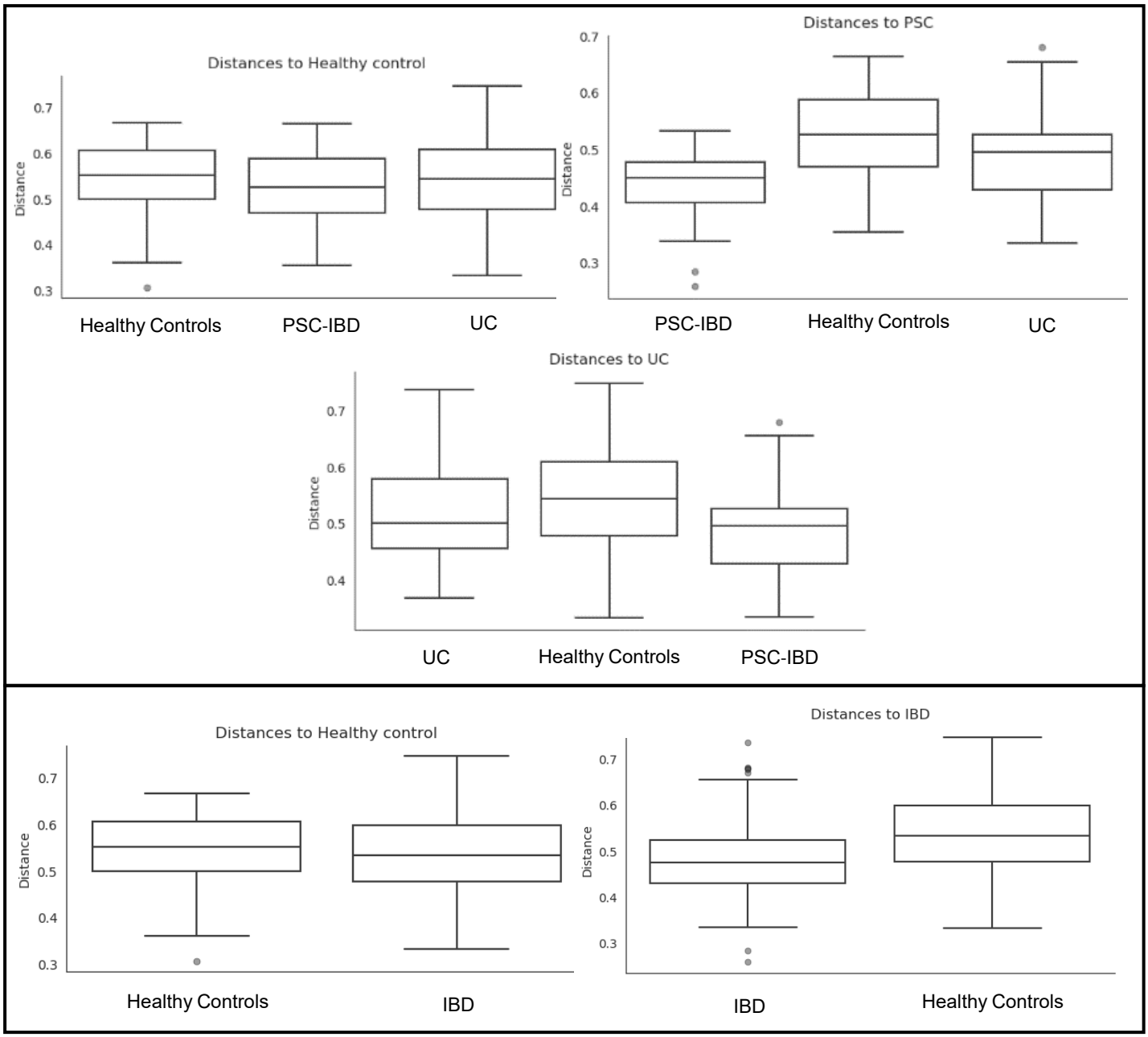


Figure 6 - 22 : Beta diversity analysis comparing cohorts

Unweighted unifrac diversity boxplots comparing beta diversity of gut microbiota between (a) three cohorts (b) Healthy controls and IBD (PSC-IBD and UC)

6.3.9 Taxa comparisons of PSC-IBD and UC versus healthy controls

To identify the relevant taxa responsible for the differences between the cohorts, we conducted LEfSe analysis. UC was characterised by a relative expansion of the phyla *Proteobacteria* and *Fusobacteria* along with reduction of phyla *Bacteroidetes* compared to HC (Figure 6 - 23).

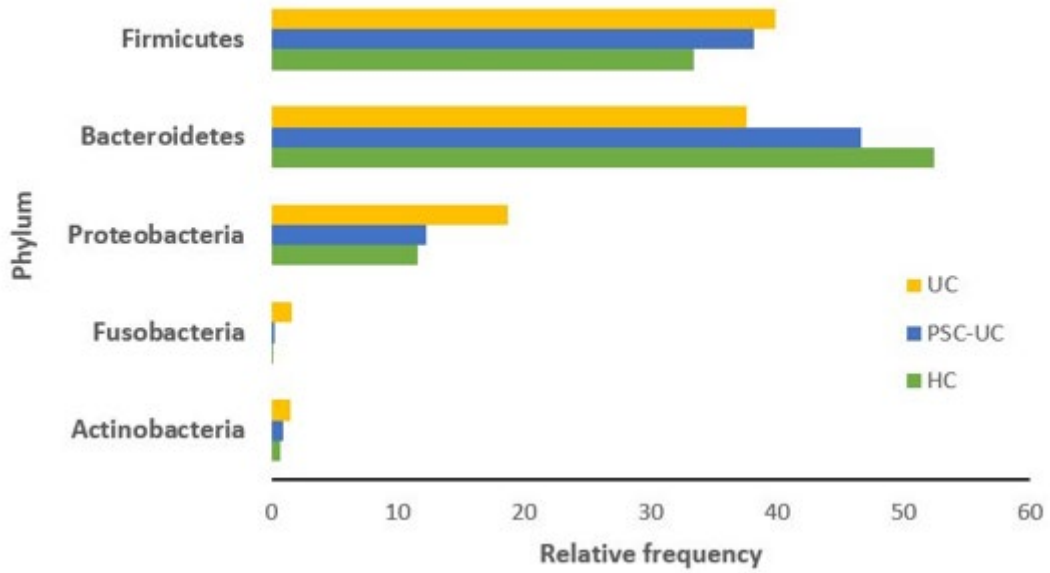


Figure 6 - 23 : Phylum level differences in the three cohorts

UC and PSC-IBD are characterised by a relative expansion of Proteobacteria and Fusobacteria and reduction of Bacteroidetes compared to HC.

PSC-IBD in comparison to HC was associated with a significant shift in taxa that included a reduction in family *Lachnospiracae* and increase in the order *Bacilli*, family *Pasteurellacae*, genus *Pseudomonas* and *Streptoccocus* and *Haemophilus parainfluenzae* species (Figure 6 – 24). UC was associated with shifts that included a significant reduction in species *Ruminoccocus sp* and an increase in genus *Anaerococcus* compared to HC.

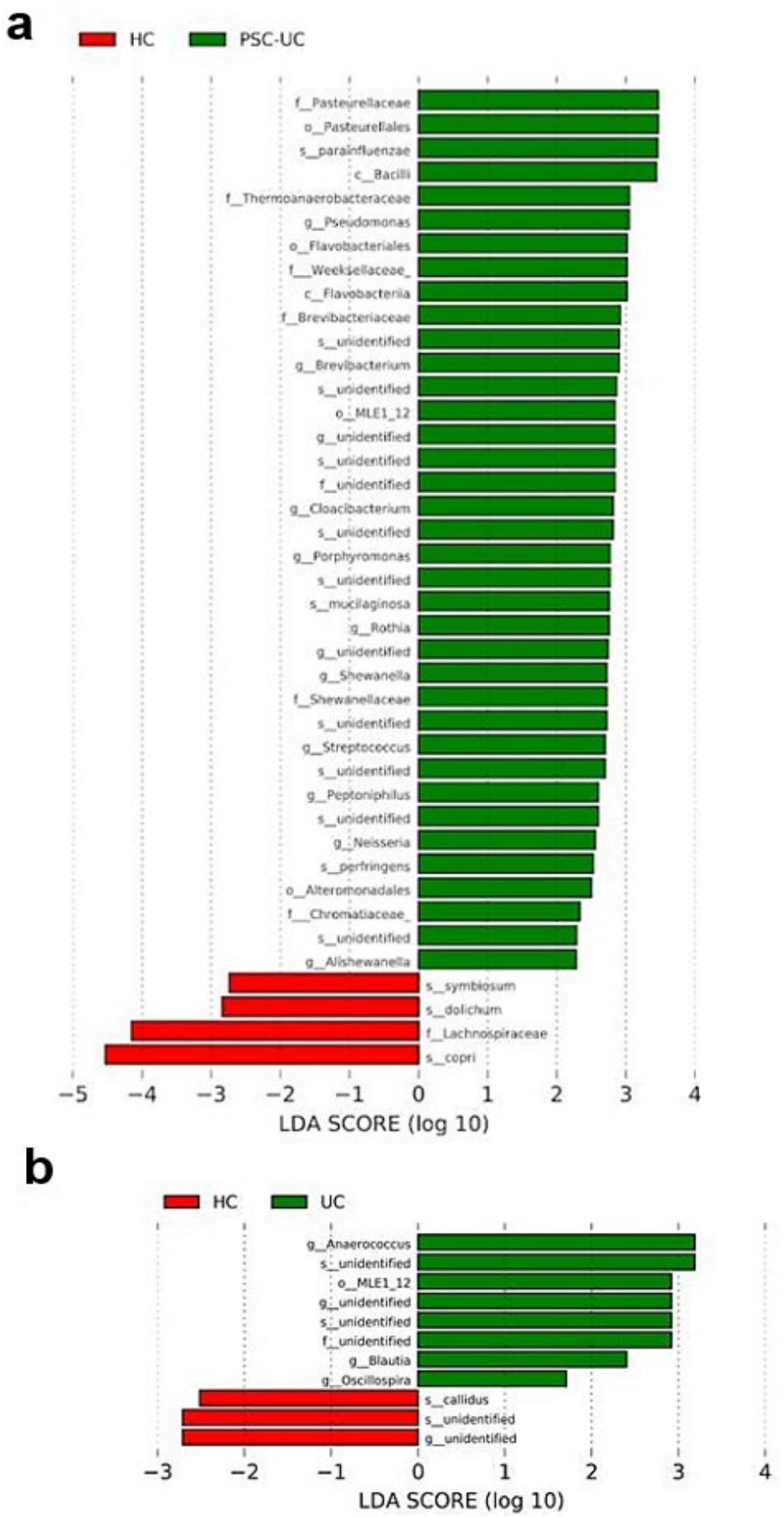


Figure 6 - 24 : Specific microbiota taxa in diseased states compared to controls.

Determined by linear discriminant analysis (LDA) effect size (LEfSe) in (a) UC vs HS and (b) PSC-IBD vs HC.

PSC-IBD vs UC

In comparison to UC, PSC-IBD was characterised by a significant difference in 50 taxa of which 24 were enriched in PSC-IBD. In PSC-IBD there was a reduction in taxa that included phylum *Lentisphaerae,* class *Gammaproteobacteria*, families *Enterobacteriacea*, *Prevotellacae*, *Paraprevotellacae*, Myxococcales and genus Streptococcus. PSC-IBD was associated with a significant increase in taxa that included the class *Bacilli*, family Shewanellaceae, genus *Staphylococcus* and species *Parvimonas sp* and *Bacteroides fragilis*. (Figure 6 – 25, 6 - 26).

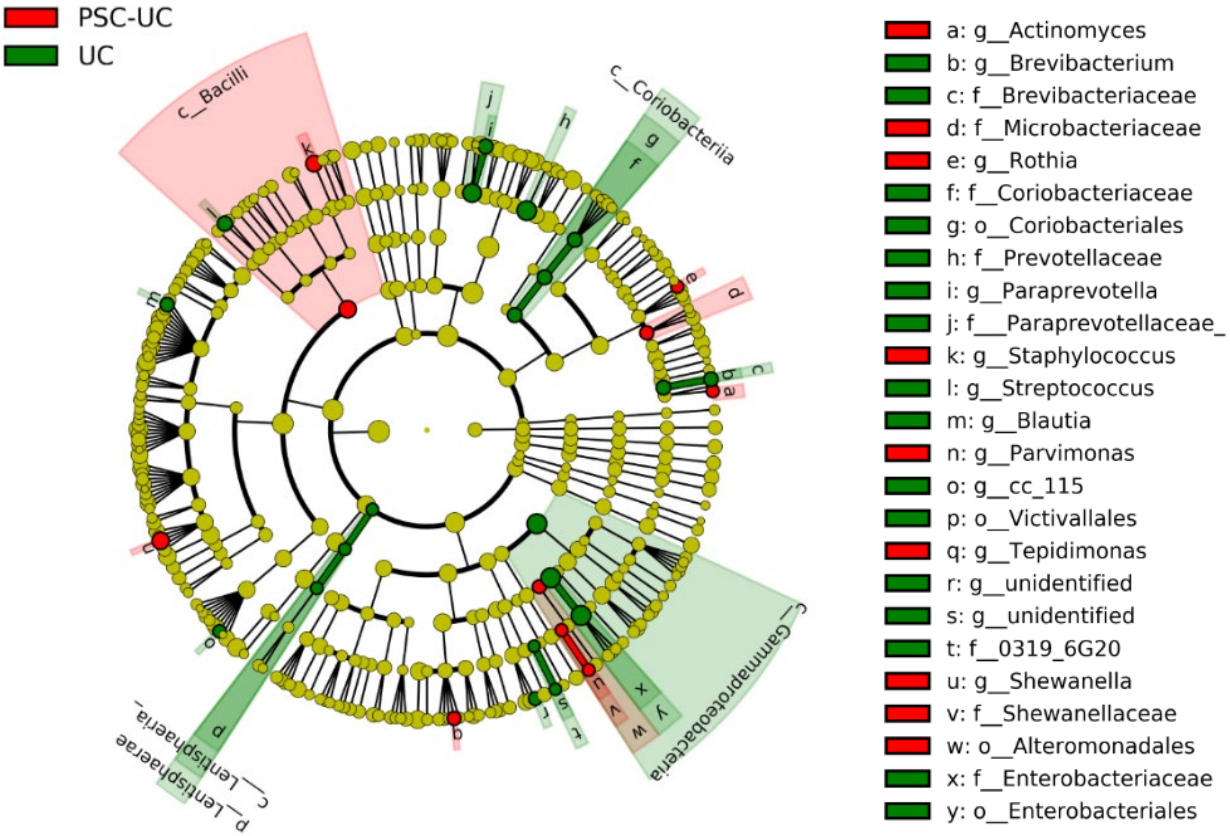


Figure 6 - 25 : Cladogram representation of the gut microbial taxa associated with PSC-IBD and UC.

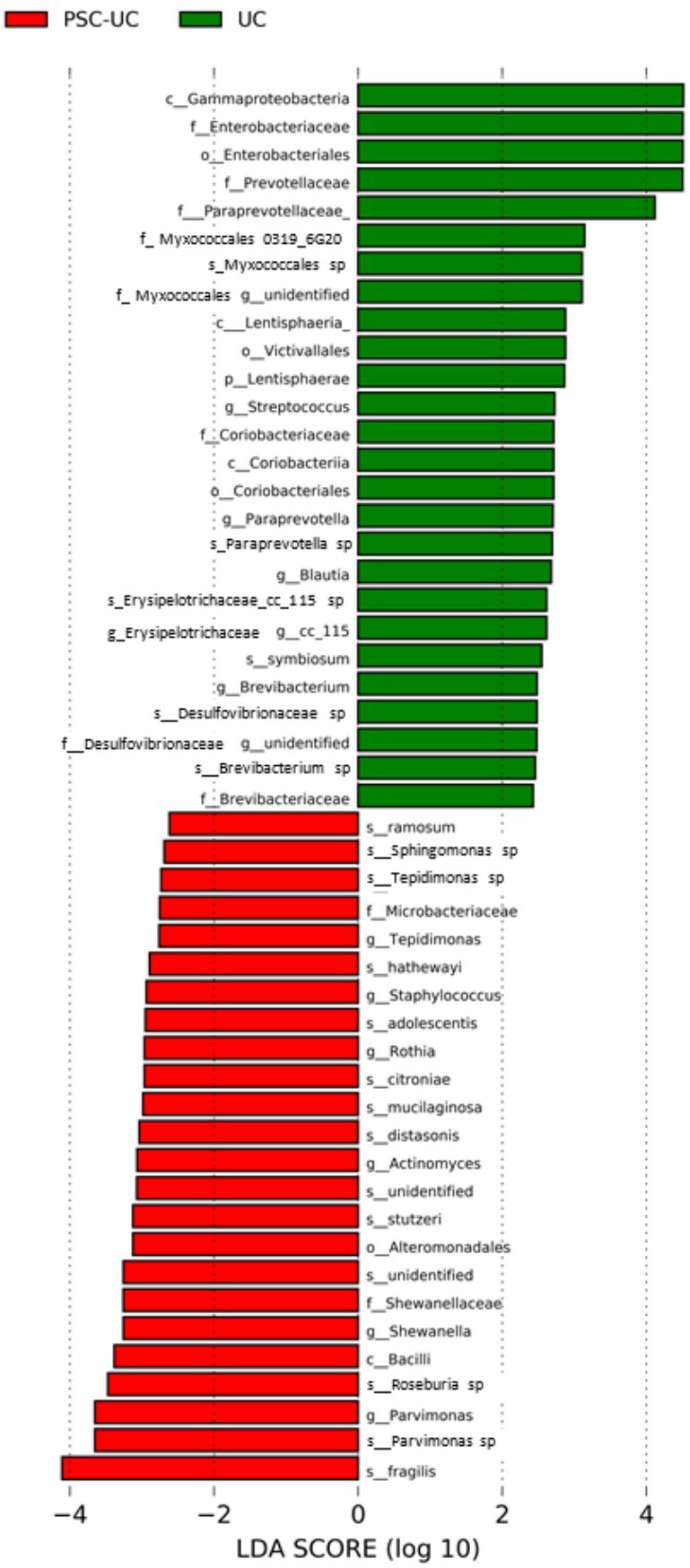


Figure 6 - 26 : Microbial taxa comparing PSC-IBD and UC by linear discriminant analysis effect size (LEfSe).

Red indicates taxa enriched in PSC-IBD and green indicates taxa enriched in UC. PSC-IBD is associated with an increased abundance of bacteria expressing bile salt hydrolase and hydroxysteroid dehydrogenases (such as Bacteroides fragilis, Roseburia spp, Shewanella spp and Clostridium ramosum) in comparison to UC.

6.3.10 Predicted metagenomic pathways in PSC-IBD compared to UC

Metagenome functional content based on the microbial community profiles obtained from the 16S rRNA gene sequences was inferred using PICRUSt2 (Figure 6 – 27). Metacyc pathway analysis revealed an increase in pathways such as glycolysis, glucose, xylose and mannan degradation.

The predicted KEGG pathways significantly enriched in PSC-IBD compared to UC included carbon metabolism in cancer, primary bile acid biosynthesis (associated with significantly higher expression of bile salt hydrolase and hydroxysteroid dehydrogenases), pentose and glucoronate interconversions, galactose metabolism and amino acid and nucleotide sugar metabolism.

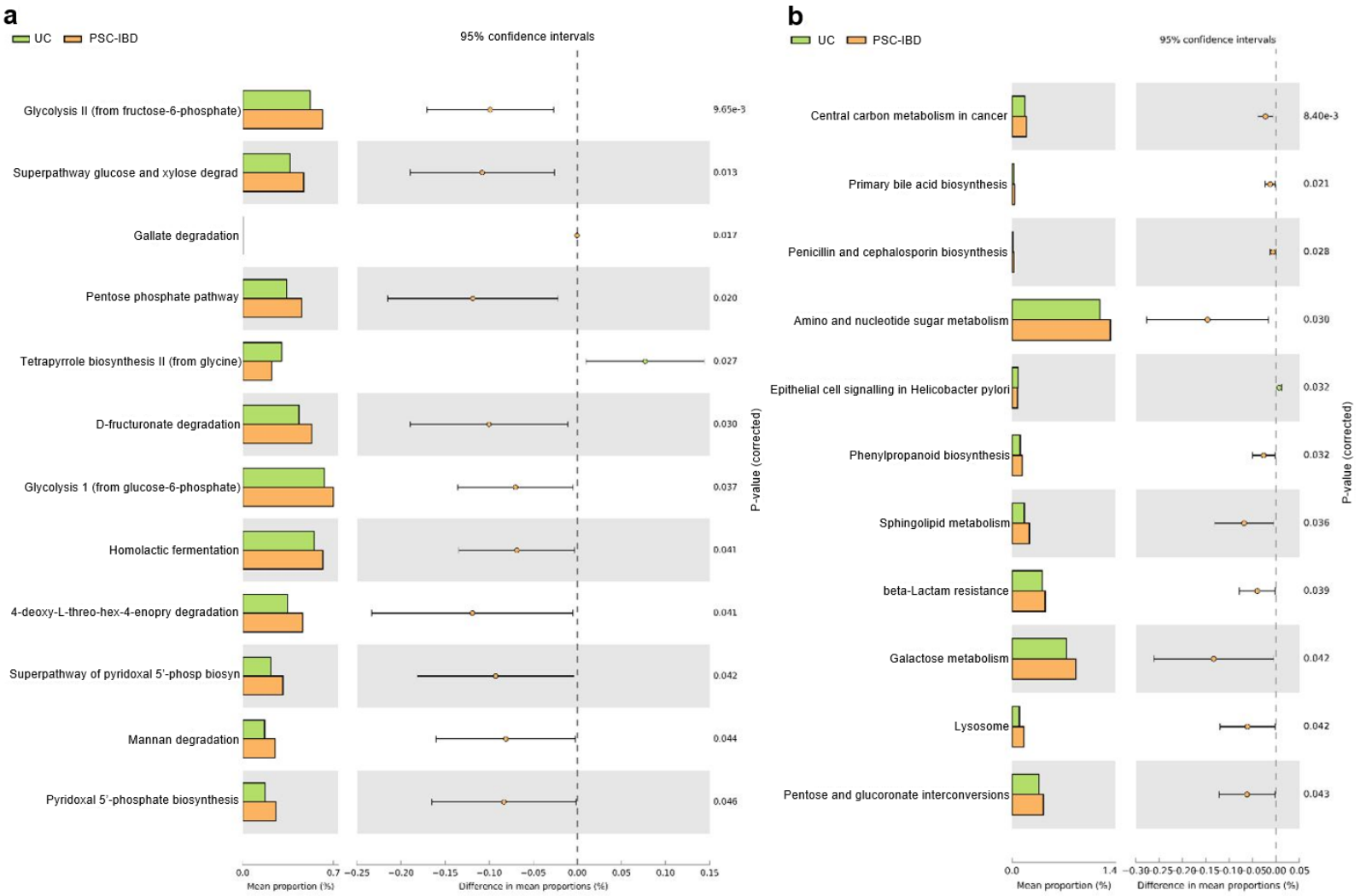


Figure 6 - 27 : Functional classification of the predicted metagenome content of the microbiota of PSC-IBD compared to UC

(a) MetaCyc pathways and (b) KEGG pathways. There is significant enrichment of primary bile acid biosynthesis (associated with significantly higher expression of bile salt hydrolase and hydroxysteroid dehydrogenases), pentose and glucoronate interconversions and galactose metabolism in PSC-IBD compared to UC.

6.3.11 Predictive analytics

Predicting disease state based on modelling of gene expression

Predictive analysis was performed using RF method and applied to the individual omics data sets. This method was used to obtain predictive performance of all the features from the transcriptomics, immunophenotype and 16S rRNA microbial profiling datasets in order to identified robust differentiating factors between the three diseases. All the genes were ranked and tested sequentially to get the optimum number of the genes that produced highest AUC values as shown in Figure 6 - 28. Those features were carried over for further network analysis. In PSC-IBD vs. HC samples, based on the AUC, we identified top 25 genes from RNA seq data with AUC value 0.97 and confidence interval (CI) of 0.80-1. In case of PSC-IBD vs. UC the number of genes were considered 50 with AUC value 0.96 with CI 0.66-1. Similarly, for UC vs. HC samples the AUC value was 0.97 with 15 genes and CI was 0.77-1.

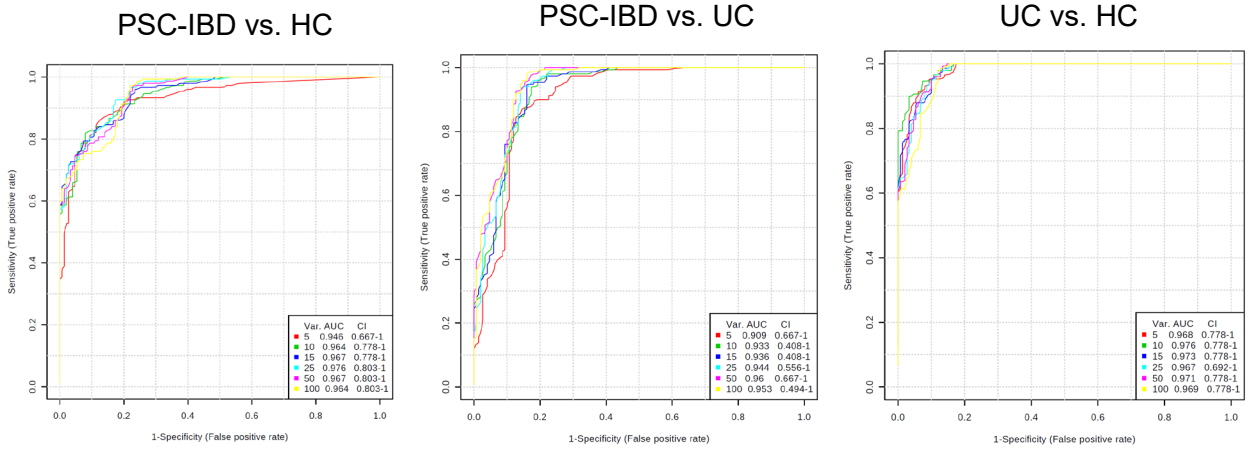


Figure 6 - 28 : Optimising gene selection for predictive modelling for each cohort comparisons.

Gene co-expression regulatory network analysis

Through network analysis we discovered that TUBB2A gene came out with highest connectivity in PSC-IBD vs UC; LYN gene was found top in the comparison with UC vs HC patients and STOM gene was found maximum connections in PSC-IBD vs, HC analysis. A list of these genes is presented in Table 6 - 2.

Table 6 - 2 : List of genes with maximum connections between the three healthy cohorts based on predicting disease modelling.

PSC-IBD vs UC	Degree
TUBB2A	78
CALU	72
USP2	70
UGT1A1	47
ASS1	44
UGT1A6	43
UGT1A9	40
UGT1A10	40
UGT1A7	39
TRIM29	38
PITX2	21
TNIP3	19
TSTA3	19
GBA3	17
UBC	16
CXCL1	15
CCL13	14
LCN2	14

PSC-IBD vs HC	Degree
STOM	68
CEP72	66
UBE2L6	40
RAB8A	21
CHCHD3	18
PSMA1	14
LCA5	8
LAX1	3
ARHGD1A	2

UC vs. HC	Degree
LYN	53
IL2RA	11
OAS2	3
IL1RN	3
NFKB1	3
RELA	3
STAT3	3

6.3.12 Assessing effects of potential confounders on gene expression

We explored the effects of specific confounders on DEGs in PSC-IBD. In patients with a liver transplant (n=3) who were also on tacrolimus only REG3A, DEFA5, DEFA6, SNORA66 and PRSS2 were upregulated and VSIG4 downregulated. In patients with UDCA (n=3) only CYP3A4 was differentially expressed (upregulated). There were no significant DEGs associated with use of mycophenolote (n=1), biologics (n=1) or cirrhosis (n=1).

6.3.13 Integrating transcriptomics, immunophenotype and 16s microbial profile

To fuse (or link) multiple data types, we used a hierarchical clustering with Pearson correlation distance measure based on PSC-IBD, UC and HC datasets. Differentially expressed genes between PSC-IBD and UC were selected based on AUC values, maximum connectivity and biological relevance. 16S rRNA microbial profiling were selected based on linear discriminant analysis (LDA) effect size (LEfSe) more than log (10) value of greater than or less than 3 between PSC-IBD and UC. Th17 and IL17 were selected as these were the two key immunological parameters that were significantly different in PSC-IBD and UC compared to HC. Clustering was observed between key genes involved in bile acid homeostasis and genes associated with cancer regulatory pathways (Figure 6 - 29).

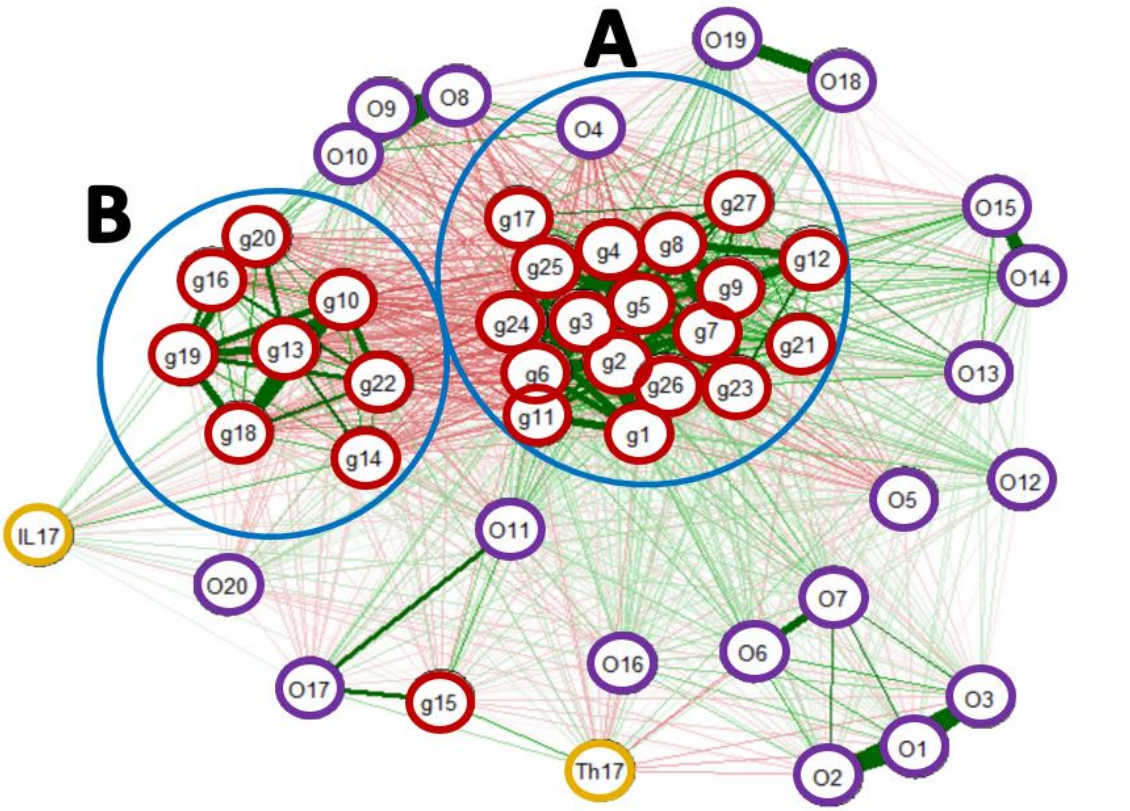


Figure 6 - 29 : Correlation networks between the mucosal transcriptome, 16S microbial profiles and immunophenotype in patients with PSC-IBD

The mucosal transcriptome is in red, 16S microbial profiles purple. Genes were selected based on supervised and unsupervised dimension reduction as described in Methods. Microbial taxa were selected based on the loading values more than log2(3) values. Th17 and IL17 expressing cells were selected as they were significantly upregulated in PSC-IBD and UC compared to HC. Red line indicates negative correlation and green positive correlation. Thickness of the line shows strength of the correlation between each features. Clusters are defined based on a high inter-feature correlation (r=0.8). Two main clusters were identified – cluster A consists of genes involved in bile acid homeostasis; cluster B demonstrates genes associated with cancer regulatory pathways.

6.4 Discussion

The clinical appearances and behaviour of PSC-IBD are distinct when compared to classical UC. This is further supported by distinctions in genetic risk, collectively supporting the concept that biologic pathways related to disease behaviour will differ. Understanding these distinctions in biology, have the potential to inform future therapeutic opportunities. For the first time and using an integrative systems biology approach we have demonstrated differences between the colonic mucosal landscape in patients with PSC-IBD and UC, and identified bile acid signalling pathways in the colon to be notably regulated differentially.

Comparative analysis of mucosal transcriptome with healthy controls confirms that the colonic phenotype in both PSC-IBD and UC is primarily immune mediated in origin. Genes and pathways associated with these genes that are responsible for multiple facets of the innate, adaptive and humoral immune response are differentially upregulated in both conditions. Furthermore, genes and pathways associated with recognized biological mechanisms including anti-microbial defence response and extracellular matrix remodeling are also differentially upregulated in comparison to healthy controls. A predominant Th17 and IL17 producing cell mucosal immunophenotype was seen in both these diseases. PSC-IBD however had a distinct mucosal transcriptomic profile compared to UC. Only 939 genes; which accounts for 70% of DEGs in PSC-IBD vs HC and 22% of DEGs in UC vs HC, had shared differential expression in PSC-IBD and UC when compared to healthy controls. Importantly comparison of gene expression profile revealed differential regulation of 1692 genes between PSC-IBD and UC. Analysis of these genes and pathways associated with this differential expression highlights key differences in physiological processes (Figure 6 - 30).

Bile acid regulation is mediated through a negative feedback mechanism as part of its enterohepatic recirculation by hormones such as fibroblast growth factor (FGF) 15 and 19. Bile acids are reabsorbed primarily by terminal ileal enterocytes and partly by colonocytes. This activates FXR (farnesoid X receptor) by upregulation of the FGF15/19 pathway which consequently leads to inhibition of hepatic bile acids synthesis through suppression of CYP7A1 enzyme. Pathway analysis in this study demonstrates that the biological processes associated with bile acid homeostasis, glucuronidation and specific metabolic functions are enriched in PSC-IBD compared to UC. Multiple nuclear receptors specifically FXR (*NR1H4*, farnesoid X receptor), PXR (pregnane X receptor) and PPAR-γ (peroxisome proliferator-activated receptor gamma) are upregulated in PSC-IBD compared to UC. As bile acids are inherently cytotoxic, these nuclear receptors, in particular FXR a bile acid-activated transcriptional factor, are activated in order to induce gene expression circuitry to protect against bile acid toxicity by shutting down expression of genes that increase influx and synthesis of bile (312-316). The intracellular concentration of bile acids is an important determinant of FXR transcriptional activity. Multiple isoforms of UDP glucuronosyltransferases (UGT) and sulfonyltransferases (SULTS) and *GBA3* (glucosylceramidase beta 3) were found to be significantly upregulated in PSC-IBD compared to UC. Unconjugated hydrophobic dihydroxy bile acids that are passively absorbed in the distal ileum and colon are converted to more hydrophilic and less toxic conjugated derivatives by UGT (via glucuronidation), SULT (via sulfation) and GBA (hydrolysis) and exported across the basolateral membrane (174, 317). These genes are expressed by activation of FXR / PPAR and functions to accelerate the neutralisation and metabolic elimination of bile acids to consequently decrease their intracellular levels (318-320). Gene expression of ileal bile acid binding protein (IBAP) and multiple FXR-dependent basolateral membrane bile acid efflux transporters including OSTα/OSTβ (organic solute transporter alpha-beta), *BSEP* (ABCB11, bile salt export pump), *BCRP* (ABCG2, breast cancer resistance protein) and *MRP3* (ABCC3, Multidrug Resistance-Associated Protein 3) are significantly upregulated in

PSC-IBD compared to UC. The position of BSEP is unclear with low levels of hepatic expression being described (321).FXR and PPAR directly control expression of IBABP and bile acid efflux transporters.

This protective positive feedback effect, possibly as a consequence of increased intracellular bile acid concentrations, significantly increases binding and exporting of bile acids through basolateral membrane transporters (322-325). Although the expression of FGF19 (fibroblast growth factor 19) a key hormone produced in response of FXR activation was unchanged, the MEP1β gene (meprin A subunit beta) was highly upregulated in PSC-IBD. This gene encodes the meprin enzyme and is produced in response to FGF19 and alters its biological activity (326). By comparison with genes associated with bile salt metabolism, genes associated with immunological response are upregulated in UC compared to PSC-IBD suggesting that the colonic phenotype in UC is derived from primarily immunological mechanisms. Similarly, immunological pathways were predominantly enriched in UC.

This differential gene expression profiles in PSC-IBD appear to suggest that these genes and pathways are significantly upregulated in order to decrease the intracellular colonic mucosal bile acid pool and reduce intracellular toxicity. It has been shown that the faecal bile acid pool is significantly reduced in PSC-IBD and is unclear whether this is due to reduced delivery of into the colon either related to cholestasis or increased bile acid clearance (237). Bile acids, through FXR activation have been shown to regulate host genes expression to promote immune responses against luminal bacteria (327, 328). It is possible that that the colonic inflammation seen in PSC-IBD is a result of the dysregulation or saturation of these mechanisms by an overwhelming increase in luminal and intracellular bile acid pool.

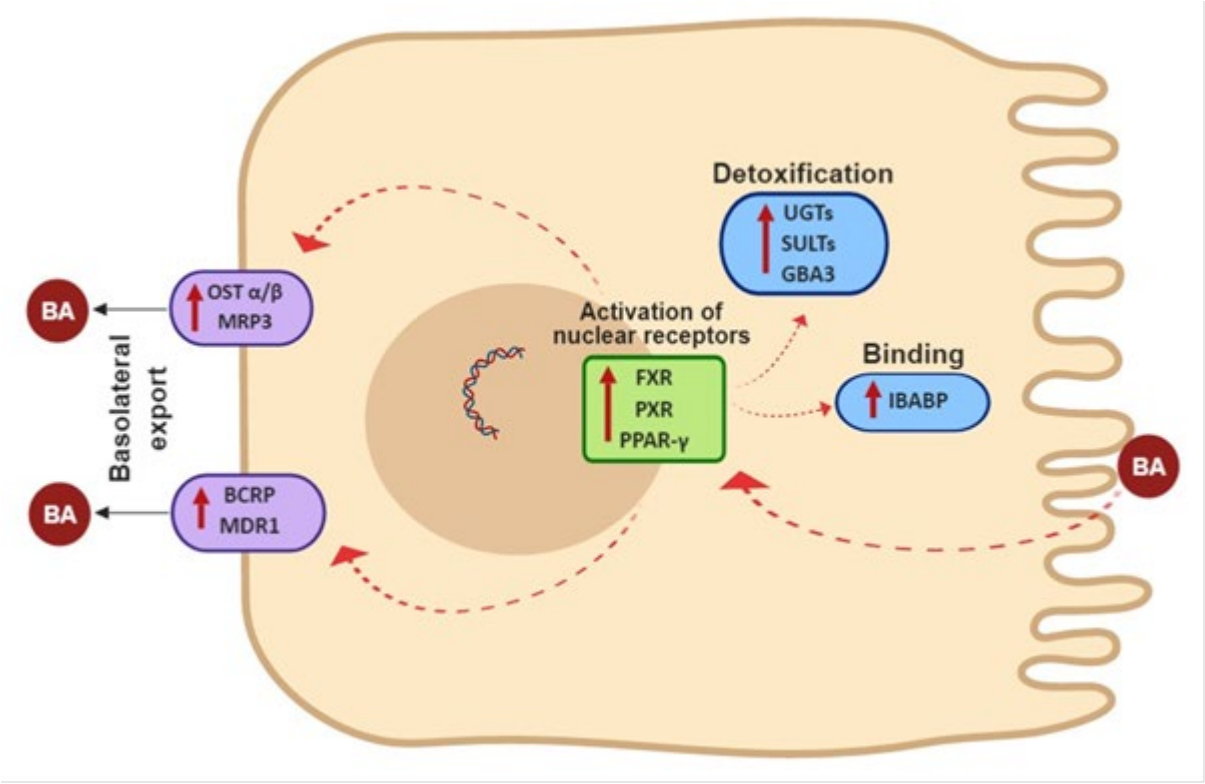


Figure 6 - 30 : Schematic representation of differential gene expression data of intracellular bile acid processes in patients with PSC-IBD compared to UC.

Red dotted arrow demonstrates positive regulation/activation of gene expression. Red bold arrow demonstrates gene upregulation. Nuclear receptors FXR, PXR and PPAR-y are activated in response to intracellular bile acids. These increase gene expression of multiple isoforms of UGTs for glucuronidation, SULTs for sulfation and GBA3 for hydrolysis for conversion of bile acids into a less toxic conjugated derivatives. These nuclear receptors also increase gene expression of IBABP to increase binding of bile acids and basolateral bile acid efflux transporters OSTa/b, MRP3, BCRP and MDR1 to promote export of bile acids. These findings are likely to be a consequence of an increase in bile acid uptake into colonic epithelial cells and suggest the possible causal role of bile acid mediated colonic inflammation in PSC-IBD

Abbreviations: BA – bile acids, FXR – farnesoid X receptor (NR1H4), PXR – pregnane X receptor (NR1I2), PPARy - peroxisome proliferator-activated receptor gamma (PPARG), UGT - UDP-glucuronosyltransferase, SULT – sulfotransferases, GBA3 - cytosolic beta-glucosidase, IBABP – ileal bile acid binding protein (FABP6), OST - organic solute transporter (SLC51A, SLC51B), MRP3 - multidrug resistance associated protein (ABCC3), BCRP – breast cancer resistance protein (ABCG2), MDR1 – multidrug resistance gene (ABCB1).

Mucosal gut microbiota profiling revealed significant distinctions in the global profiles between PSC-IBD, UC and HC. These results, consistent with our previous findings and published literature, confirm that the dysbiosis in PSC-IBD is independent from UC. The dysbiosis seen in PSC-IBD appears to be associated with changes in bile acid metabolic pathways. Patients with PSC-IBD had significantly higher abundances of species *Bacteroides fragilis*, *Roseburia spp*, *Shewanella spp* and *Clostridium ramosum* in comparison to UC. These bacteria express bile salt hydrolase (BSH), an enzyme that catalyses the deconjugation of bile acids in the gut (329-332). These bacteria have also been shown to express hydroxysteroid dehydrogenases which results in biotransformation of primary to secondary bile acids (333, 334). Inferred metagenomics (PICRUSt2) further corroborated significant enrichment of BSH and hydroxysteroid dehydrogenases in gut microbiota profiles of patients with PSC-IBD compared to UC (and HC) in our dataset. *Sphingomonas sp*, a bacterium that expresses amine oxidase, was found to be upregulated in PSC-IBD compared to UC. This enzyme is associated with aberrant homing of gut lymphocytes to the liver and is strongly considered to be a mechanism underlying the gut liver inflammatory axis seen in PSC-IBD (161, 335). FXR has been shown to modulate gut microbiota and mice deficient in FXR on high fat diet had a higher abundance of Firmicutes and lower abundance of Bacteroidetes in comparison to wild type mice possibly associated with a reduced bile acid metabolising capacity (336, 337). Although as yet unclear, it is likely that the dysbiosis is both a consequence and cause of the alteration of biliary homeostatic gene expression profiles and the colonic and biliary disease seen in PSC-IBD (338). Although limited by very small numbers, no differences in gut microbiota profiles were seen between pre and post liver transplant PSC patients.

Our study demonstrates for the first time that the colonic mucosal immune response in PSC-IBD characterised by a significantly higher Th17 cell and IL-17 producing CD4 T cell population

compared to HC. These findings were similar to those seen in the UC cohort, however patients with PSC-IBD also have a corresponding lower Th1 cell population and higher IL17/IFNγ dual producing CD4 cells population compared to controls. IL-17 producing Th17 cells are often present at the sites of chronic tissue inflammation in multiple autoimmune diseases (100, 227, 339). A recent study demonstrated that transfer of IBD microbiota into germ-free mice numbers of intestinal Th17 cells and exacerbated colitis in a T cell transfer Rag1 knockout mouse model (105). This has led to the inference that Th17 cells are the critical immunological drivers of inflammation in autoimmune diseases and are likely to be induced by specific components of gut microbiota. The role of bile acids in directly mediating host immunity by controlling Th17 cell response has been recently explored (340). Bile acids in tissues have been shown to upregulate expression of IL23, which in turn maintains Th17 expansion and promotes the production of IL17 (341). This has been shown to synergistically enhance bile acid induced production of inflammatory mediators in mice with obstructive cholestasis. Furthermore, we found significant upregulation of CYP27A1 expression in PSC-IBD compared to UC. This is a key enzyme that functions in generating oxysterols, which are key intermediates for bile acid synthesis and have been shown to function as RORγt ligands to drive Th17 cell differentiation (234). Our findings highlight the need to explore the role of key players such as the gut microbiome and bile acid in mucosal T cell homeostasis in PSC.

We found multiple other pathways to be significantly upregulated in PSC-IBD. Genes and pathways associated with cancer regulation such as DNA damage repair and checkpoints, p53 signalling pathway, mitosis transition phases and APC/CCdc20 mediated the degradation of Cyclin A were significantly down regulated in PSC-IBD compared to UC. Network analysis revealed the gene TUBB2A to be a potential key mediator in gene expression profiles in PSC-IBD compared to UC. We have previously shown that methylation a component of this gene,

beta-tubulin TUBB6, significantly correlated with the presence of and accurately discriminated between dysplasia and nondysplastic tissue (342). Interestingly p-ANCAs in autoimmune liver diseases are directed against a further component of the beta-tubulin family, TUBB5 cross reacting with the bacterial protein FtsZ (343). This was suggested to reflect an abnormal immune response to gut bacteria and is presumed to be a potential mechanism in autoimmune liver disease. PRAC1 gene was found to be 8 log fold down regulated in PSC-IBD and expression of this gene has been shown to be associated with reduced susceptibility for right sided cancers. As PSC is a risk factor for colon cancer in patients with UC (with higher rates of right sided cancers) these findings are of great interest and deserve further exploration into possible underlying mechanisms.

One of the major strengths of our study is that this is the first multi-omics to date that has attempted to unravel disease mechanisms by integrating mucosal transcriptomics, immunophenotyping and mucosal microbial profiling. Through this approach we have demonstrated that consistent with clinical phenotype and GWAS datasets, the biological mechanisms underlying colonic pathology appear to be distinct in PSC-IBD compared to UC. We have made novel observations and proposed disease processes that need validation using through ex vivo experiments such as co-culture of colonic organoid with bile acids and/or specific gut bacteria. Through cell deconvolution techniques we were able to ascertain that the transcriptomic findings were not a result of significant differences in cell subset populations apart from a slight increase in dendritic cells in PSC-IBD compared to HC. This technique does have limitations and future studies should strongly consider using single cell RNA-sequencing in exploring the function of colonic epithelial cells in bile acid homeostasis (344).(50) Power calculations for multi-omics studies that include RNA-sequencing experiments are not fully established. However, between 6 to 12 biological replicates for each cohort have been

recommended (345, 346). Consequently, sample sizes are generally decided based on cost implications and feasibility of recruitment especially of rare orphan diseases with strict exclusion criteria. Despite our relatively small sample size, we were able to infer significant biological differences with high confidence between the three cohorts. This was further demonstrated by predictive modelling of disease phenotypes using Random Forest (RF) machine learning method to overcome small “n” and large “p” problem in the literature. One of the advantages of using machine learning over traditional methods are that they are non-parametric in nature and do not rely on statistical assumptions. In this we combined two approaches: data driven approach in order to facilitate mechanistic understanding of the pathways in an unbiased way together with hypothesis driven approach (347, 348). We recognise that a significant number of genes that we have explored are likely to have multiple yet unknown or less established functions and consequently pathway analysis is therefore limited in its approach. Three of the patients with PSC-IBD were on UDCA and this theoretically can change the expression of FXR and alter bile acid homeostatic pathways in the intestine. Gene expression analysis revealed that CYP3A4 was the only gene that was differentially regulated in PSC patients and was increased in those on UDCA compared to those who were UDCA naïve. Furthermore, UDCA has not been shown to activate FXR expression but rather antagonise it as demonstrated by a randomised controlled pharmacodynamic study and in silico FXR binding experiments (349-351). It is therefore unlikely for UDCA to have had an impact on the gene expression profiles seen in PSC patients. Although the median bilirubin was in PSC-IBD was 24.5 ummol/l, only two patients were jaundiced (bilirubin < 80 ummol/l) and one of whom had Child Pugh A cirrhosis. We recognise that this may potentially impact the gene expression profile however subgroup analysis did not show significant differences in DEGs as a consequence of this. We also recognise that cause or effect of cholestasis would be difficult to establish from our data as the very nature of PSC means cholestasis is its primary manifestation. However, to further delineate this a control arm with UDCA naïve patients with cholestatic liver disease and without colonic

inflammation would need to be included. However, the feasibility of identifying such patients undergoing colonoscopy outside a research setting is challenging. Through an additional experiment we were able to show that gene expression patterns are stable and similar in a healthy individual regardless of site of biopsy (i.e. right, transverse or left colon). Although all the biopsies were standardised to the sigmoid colon, if this stability of gene expression profile also applies to PSC-IBD and UC is not clear. Furthermore, it remains to be established how the colon compares with ileal bile acid homeostatic mechanisms in health and disease. Finally, it remains to be established how the colon compares with ileal bile acid homeostatic mechanisms in health and disease and the role of diet as a modifier of the PSC-IBD transcriptome, epigenome and microbiome. Future studies should strongly consider dietary evaluation and investigating tissue, faecal and serum bile acid profiles.

6.5 Conclusions

Our study is the first to present the colonic mucosal landscape in PSC-IBD. By using a systems biology approach that integrates the mucosal transcriptome, immunophenotype and mucosal microbial profile we able to demonstrate the colonic inflammation in PSC-IBD, like UC, is immune mediated when compared to healthy controls and presents as a predominant Th17 and IL17 producing CD4 cell response. PSC-IBD however, is transcriptomically distinct to UC, with dysregulation of genes associated with multiple bile acid homeostatic partly mediated by differences in gut dysbiosis. These findings cautiously support that the colonic mucosal immune mediated inflammation in PSC-IBD is possibly contributed by colonic mucosal bile acid toxicity.

6.6 Data availability

RNA-Seq data for this study have been deposited in ArrayExpress database with the accession code E-MTAB-7915. 16S rRNA data has been submitted to the Sequence Read Archive (SRA) under accession reference PRJNA533720.

6.7 Acknowledgements

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CHAPTER 7

Conclusions

The human gut is colonized by trillions of microorganisms consisting of over two thousand species. These have co-evolved with the host to form robust a symbiotic relationship in health. The presence of large numbers of these symbionts near the epithelial surface of the intestine however poses a significant challenge to the host in order to prevent inappropriate activation of inflammatory responses while conserving its capability to mount immune responses to pathogenic organisms. In chronic immune mediated inflammatory diseases such as IBD and PSC-IBD there appears to be a breakdown and loss of these intestinal homeostatic mechanisms for reasons that have gradually becoming more evident over the last few years. Through a detailed systems biology approach, I have attempted to explore and highlight a few of these key novel mechanisms in my PhD. In this chapter I will aim to summarise how findings from my work can help drive the direction of future research in this field.

The incidence of inflammatory bowel disease (IBD) however continues to rise in North America and Western Europe and is rapidly emerging in populations in Asia. It is anticipated that within certain part of UK, IBD will affect nearly 1% of the population by 2030 (352). Although genetic studies have identified over than 200 loci that regulate IBD risk, a relatively stable gene pool over the last three decades at least, far from explain this epidemic rise in incidence of IBD. This progressive geographical and temporal changes in epidemiology of IBD imply that environmental factors are likely have a major role in inducing or modifying manifestation of disease. Epidemiological studies provide important clues to potential environmental factors predisposing to the development of IBD including use of antibiotics in childhood, Western diet and lifestyle measures (353). Each of these factors is independently associated with an imbalance in gut microbiota. Consequently, a reduced diversity of the population of microbial species as well as reduced abundances of short chain fatty acid (SCFA) producing gut bacteria appears to be a characteristic and consistent feature of IBD. Although the pathogenesis of IBD

is poorly understood, numerous lines of evidence suggest that the disease is caused by a confluence of genetic and environmental factors that alter gut homeostasis, thereby triggering immune-mediated inflammation in genetically susceptible individuals. With considerable evidence demonstrating a complex dynamic and bidirectional relationships between gut microbiota and immune mediated inflammatory responses in IBD, therapeutic manipulation of gut microbiota by FMT appears to be an attractive strategy.

Our understanding of mechanisms by which FMT in modulates colonic inflammation have been limited to animal models of IBD studies with human studies exploring largely changes in gut microbial profiles and peripheral immune cell changes. Through my PhD, I was able to demonstrate that patients who respond to FMT have a significant increase in gut homing regulatory cells, a reduction in proinflammatory Th17 cells, significant downregulation of anti-microbial defence (associated with reduction in anti-microbial peptide gene expression) and proinflammatory immunological pathways and an increase in butanoate and propanoate metabolic pathways. Due to clinical trial regulatory constraints, I was not able to analyse changes in recipient microbial strains associated with response and restoration of gut immunological homeostasis as part of my thesis.

FMT, however, is like no other pharmacological drug. The underlying active constituent(s) of FMT as well as mechanisms that underpin its therapeutic success are as yet unknown. In addition to nearly 1011 bacteria per gram of wet stool, FMT also consists of bacteriophages, fungi, host and microbiota derived metabolites, antimicrobial peptides and immunoglobulins amongst other ‘unknowns’ (354). At least in Clostridium difficile infections, it appears that these non-bacterial components are equally important, with similar response rates seen in faecal

filtrate and stool derived metabolite studies (355). In IBD, however, bacteria are a critical component associated with response to FMT. Gut microbiota analyses from RCTs exploring FMT for the treatment of UC demonstrate that clinical response to FMT in patients with active UC is associated with enrichment of specific Clostridium clusters that include the SCFA-producing families such as Roseburia intestinalis, Ruminococcaceae and Lachnospiraceae (127, 137, 138). It however remains unclear if this enrichment is due to donor derived engraftment of microbial strains or a shift in the patient's own gut microbial community with a study observing that enrichment of a bacteriophage that targets gut Lactobacillus, Escherichia, and Bacteroides populations following FMT was associated with a lack of clinical response to FMT (132). Baseline microbiota and metabolite predictors of response have been explored in the FOCUS trial in which forty infusions of pooled multi-donor FMT were given over an 8-week period to patients with UC. FMT treated patients who achieved the primary outcome (clinical remission with endoscopic remission or response) demonstrated a higher stool and gut mucosal microbial diversity at baseline as well as during and after FMT therapy compared with those who did not achieve the primary outcome. The authors observed that increased relative abundances of Eubacterium hallii, Roseburia inulinivorans, Eggerthella species and Ruminococcus bromii were the strongest predictors of achieving the primary outcome (137). On the other hand, a range of microbial taxa including Fusobacterium, Haemophilus, Escherichia, Megamonas, Clostridium XIVa and Prevotella were associated with failure to achieve the primary outcome. Furthermore, they demonstrated that specific baseline recipient metabolic profiles such as greater abundances of 5 –aminovalerate, sphingomyelin and gulonate was predictive of response to FMT. Through a post-hoc exploratory analysis of fungal communities they described that high Candida abundance pre-FMT was associated with a clinical response, and hypothesised that Candida may possibly contribute to the establishment of a niche more permissive to engraftment of FMT (356).

As gut microbiota compositions vary within healthy individuals, it is highly likely that stool donor characteristics are equally important predictors of response to FMT. Prior studies have observed that FMT derived from specific donors, often referred to as 'super donors' in popular science, appears to be associated with favourable outcomes. Moayyedi and colleagues demonstrated that FMT derived from a specific donor - donor B, was associated with a greater treatment success in UC. Stool microbial profiling showed that donor B had greater enrichment for Lachnospiraceae and Ruminococcus with a trend for responders having microbiota that was more similar to donor B than non-responders. Similarly, the FOCUS trial demonstrated that within batches of FMT, Bacteroides fragilis and Bacteroides finegoldii was signficiantly higher in abundance in effective batches. In contrast higher abundances of Clostridium XIVa, a taxon associated with negative primary outcome, was associated with ineffective batches. Whilst accepting that a donor specific effect may exist with regards to the efficacy, the evidence is not there as yet for UC (357).

Collectively these finding may help explain reasons for the loss of intestinal mucosal immunological homeostasis seen in IBD. It would however be naïve to assume that the aetiopathogenesis of all inflammation is the same. One such phenomenon that exists in this field is if the IBD we see in PSC-IBD is the same and if it has similar triggers highlighted above. GWAS studies have shown minimal overlap between IBD and PSC-IBD susceptibility risk loci, and far from account for the 70% coexistence of IBD in patients with PSC. In addition, gut microbial profiles in patients with PSC-IBD have been consistently shown to be different to that seen in patients with conventional IBD. Equally importantly the clinical phenotype and behaviour colonic inflammation in PSC-IBD appears to be distinct to that seen in patients with UC or Crohn's disease. This begs to question if all inflammation is the same and if all forms of IBD are

the same, or if all we are seeing is very simply a state of chronic inflammatory processes that have different multifactorial causes. Understanding and identifying the cause or drivers of these inflammatory process would help development of disease specific therapeutic targets.

Through work done in my PhD, I demonstrated that PSC-IBD and UC have a similar immunological profile, with a significant increase in pro-inflammatory Th17 and IL17 producing CD4 cells. However, the mechanisms associated with colonic inflammation in PSC-IBD appeared to be distinct and linked to disturbances in bile acid metabolism. Importantly, we show that in PSC-IBD gut mucosal expression of the nuclear bile acid receptor farnesoid X receptor (FXR) was upregulated, in addition to intracellular bile acid binding proteins and multiple basolateral bile acid efflux transporters(358)(358)(353)(352)(351). The reasons for this are not known, but potentially it represents a protective response to limit epithelial damage from bile acid toxicity. Indeed, patients with PSC-IBD show significant upregulation of CYP7A1 (an enzyme mediating the rate-determining step of bile acid synthesis), with generation of key intermediates necessary for bile acid function. The latter includes retinoic acid receptor (RORγt), which has been shown to drive Th17 cell differentiation and mucosal inflammation. By exploring mucosa-adherent gut microbiota, I was able to demonstrate that gut microbiota in these patients with PSC-IBD compared to UC alone and healthy controls. Moreover, the dysbiosis in PSC-IBD is associated with changes in bile acid metabolism; specifically an abundance of species involved in the dehydroxylation of primary bile acids, and which express the enzymes bile salt hydrolase and hydroxysteroid dehydrogenase, that lead to the generation of oncogenic secondary bile acids in the gut (358).

Taken together, these findings suggest that mucosal inflammation in PSC-IBD is associated with colonic bile acid disturbances, secondary to pathogenic alterations in the gut microbiota. A recent seminal study by Song et demonstrated that genetic modification of BA metabolic pathways in individual gut symbionts altered Treg cell populations in mouse colonic tissue and was modulated by key bile acid sensing nuclear receptors (236). The study by Nakamoto et al. provides further evidence for a possible mechanism of action of specific bacterial species in PSC-IBD, namely Enterococcus gallinarium, which leads to increased gut permeability, liver inflammation, and confirms an increased proportion of Th17 cells in the gut and the liver (338, 359). Notably, pilot clinical studies using the orally administered, non-absorbable antibiotic vancomycin demonstrate a statistically significant reduction in alkaline phosphatase values (the biochemical hallmark of PSC disease activity), in addition to the PSC Mayo Risk Score (MRS) and total serum bilirubin (360). More recently, study of patients with PSC and active colitis demonstrated how vancomycin was able to induce and maintain IBD remission in all eight patients treated with an 8-week course (361) in addition to a reduction in secondary bile acid concentration in stool (362).

With the rapidly expanding therapeutic options for IBD with clinical remission rates of between 15% and 35% and lack of any effective medical options for PSC there is a growing need to move towards personalised therapy by understanding the underlying triggers and mechanisms of disease. Carefully conducted open label mechanistic studies would help understand and unravel causative and potentially modifiable factors. There is a drive towards rational donor selection strategies based on both donor characteristics as well as donor-patient matching. This involves selecting a donor or donor FMT that have greater abundances of specific beneficial taxa that may be missing in a patient with UC or PSC-IBD. Furthermore, optimisation of stool donors may be implemented through a period of a prescriptive dietary modification prior to stool

donation. This, in conjunction with prebiotic supplementation of patients in order to create a favourable niche that promotes engraftment and growth of beneficial donor strains are novel approaches that deserve to be explored further.

In order to understand what this actually means we now need to move towards carefully conducted studies that explore causation. Clinical studies investigating the therapeutic use of FMT in the treatment of IBD or PSC-IBD should now adopt a focused mechanistic approach towards exploring changes in host immunology, host bile acid pathways and transcription in relation to changes in the gut metagenome, metatranscriptome and metabolome. This includes understanding immune cell subset dynamics along with exploration of interrelationships within the spatial context of the tissue microenvironment. Furthermore, single cell sequencing of immune subsets and immune cell receptors, particularly in patients with IBD where there is a clear gut mediated immunological trigger would help delineate tissue specific adaptation, transcriptional dynamics and plasticity potentially towards a Treg phenotype in response to FMT. Finally, undertaking a systems biology host-microbial ‘omics’ integrative approach has the clear potential of identifying microbiota derived mediators in driving immunoregulatory pathways.

Links to Appendices

Appendices A - STOP-Colitis Differential gene expression dataset (Excel Spreadsheet) - Differentially expressed genes associated with treatment with FMT - shorturl.at/ehnpY

- Sheet 1 - All participants - Week 8 vs Baseline
- Sheet 2 - Responders - Week 8 vs Baseline
- Sheet 3 - Non-responders - Week 8 vs Baseline
- Sheet 4 - Week 8 - Responders vs Non-responders

Appendices B - STOP-Colitis Transcriptome pathway analysis (Excel Spreadsheet) - Differentially regulated gene ontology biological processes and KEGG/Reactome pathways associated with treatment with FMT - shorturl.at/ehnpY

- Sheet 1 - All participants - Week 8 vs Baseline - GO pathways
- Sheet 2 - All participants - Week 8 vs Baseline - KEGG/Reactome pathways
- Sheet 3 - Responders - Week 8 vs Baseline - GO pathways
- Sheet 4 - Responders - Week 8 vs Baseline - KEGG/Reactome pathways
- Sheet 5 - Non-responders - Week 8 vs Baseline - GO pathways
- Sheet 6 - Non-responders - Week 8 vs Baseline - KEGG/Reactome pathways
- Sheet 7 - Week 8 - Responders vs Non-responders - GO pathways
- Sheet 8 - Week 8 - Responders vs Non-responders - KEGG/Reactome pathways

Appendices C - PSC-IBD transcriptome (Excel Spreadsheet) - Differentially expressed gene dataset in PSC-IBD omics study - shorturl.at/agBG5

- Sheet 1 - PSC-IBD vs HC
- Sheet 2 - UC vs HC
- Sheet 3 - PSC-IBD vs UC

Appendices D - PSC-IBD transcriptome (Excel Spreadsheet) - Differentially regulated gene ontology biological processes and KEGG/Reactome pathways in PSC-IBD omics study - shorturl.at/agBG5

- Sheet 1 - PSC-IBD vs HC - GO pathways
- Sheet 2 - PSC-IBD vs HC - KEGG/Reactome pathways
- Sheet 3 - UC vs HC - GO pathways
- Sheet 4 - UC vs HC - KEGG/Reactome pathways
- Sheet 5 - PSC-IBD vs UC - GO pathways
- Sheet 6 - PSC-IBD vs UC - KEGG/Reactome pathways

Publications during PhD

Publications arising from thesis (* denotes joint first author)

1. **Quraishi MN**, Yalchin M, Blackwell C, Segal J, Sharma N et al. STOP-Colitis pilot trial protocol. A prospective, open-label, randomised pilot study to assess two possible routes of Faecal Microbiota Transplant delivery in patients with ulcerative colitis. **BMJ Open.** 2019 Nov 11;9(11):e030659. doi: 10.1136/bmjopen-2019-030659.

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