Clinical, microbiological and metabolic features of patients with Crohn's disease and intestinal failure

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Statement of Originality

I can confirm that Chapter 1 – 4 are my own work. For Chapters 5 and 6, Maria Valdivia and the National Phenome centre helped by conducting the mass spectrometry experiments. The nuclear magnetic resonance experiments were conducted by Jose Serrano Contreras. The microbiota work was carried out by Nathan Danckert. All the scientists also helped with data statistics. Laura Martinez-Gili, statistician, also provided guidance on the statistics. I have referenced or acknowledged any work that has been derived from other sources or other people.
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Copyright declaration

Abstract

The pathogenesis of inflammatory bowel disease (IBD) involves the interaction between an individual's genes, gut microbiota and environmental factors. The Crohn's disease (CD) microbiome is characterised by a reduction in *Firmicutes* and an increase in *Proteobacteria*. The CD metabonome consistently shows a reduction in secondary bile acids (SBAs) and an increase in stool and serum primary bile acids (PBAs). Very few studies have examined the multiomic profile in patients with Crohn's disease-intestinal failure (CD-IF).

I present two retrospective case-control studies and one prospective multiomics study. I identified the following risk factors for the development of IF in patients with CD: female gender, ileocolonic involvement, penetrating disease and non-exposure to biologic medications. Patients with CD-IF are twice as likely to be in remission compared to patients with CD (without IF) and the strongest factor associated with active disease, was the presence of a surgical anastomosis to colon in continuity compared to those with an end enterostomy.

For the multiomics study, stool, serum and urine samples from CD-IF patients were analysed. 16s rRNA sequencing demonstrated an expansion of aerotolerant and facultative anaerobic microorganisms, with a decrease in obligate anaerobes; the CD-IF gut luminal environment has a higher oxygen content. Metabonomic profiling showed a higher serum and faecal cholic acid and chenodeoxycholic acid in active disease compared to remission; this is in keeping with the theory that loss of bowel length leads to loss of farnesoid X receptor (FXR) and Fibroblast growth factor 19 (FGF19) and an increase in hepatic synthesis of PBAs. Expectedly, an almost absence of serum SBAs was observed in CD-IF. Multiomics changes seen with active disease could be a cause or a consequence of disease activity, or secondary to the anatomic changes or changes in diet and parenteral nutrition.

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List of abbreviations

BL: bowel length

CA: cholic acid

CD: Crohn's disease

CD-IF: Crohn's disease-intestinal failure

CDCA: chenodeoxycholic acid

CLR: center log-ratio data transformation

CRP: C-reactive protein

CV ANOVA: cross validated analysis of variance

Glyc: *N*-acetylglycoprotein

IBD: inflammatory bowel disease

IF: intestinal failure

LC-MS: liquid chromatography-mass spectrometry

MCCV PLS DA: Monte carlo cross validation partial least squares – discriminant analysis

NMDS: Non-metric multidimensional scaling

NMR: nuclear magnetic resonance

MTBE: Methyl tert-butyl ether

PBA: Primary bile acid

PC: phosphocholine

PCA: principal component analysis

PLS: partial least squares

PERMANOVA: permutational multivariate analysis of variance

SBA: secondary bile acid

UC: Ulcerative Colitis

UPLC-MS: ultra performance liquid chromatography mass spectrometry

Chapter 1 Introduction

1.1 Background

1.1.1 Crohn's disease overview

Inflammatory bowel disease (IBD) consists of Crohn's disease (CD), Ulcerative Colitis (UC) and Indeterminate Colitis (IC). Crohn's disease (CD) is a chronic relapsing and remitting inflammatory bowel disease that causes transmural inflammation, which can occur anywhere along the gastrointestinal tract and was first discovered in 1932 by the identification of a collection of patients with a regional ileitis(Crohn, Ginzburg and Oppenheimer, 1932). The disease course ranges from mild to aggressive; with progressive inflammation and structuring or penetrating behaviours sometimes requiring surgery. CD has a much higher incidence in the West than in Asian countries with incidences of 29.3 and 0.54-3.44 per 100,000 respectively (Ng, Wong and Ng, 2016). The prevalence of Crohn's disease is rising worldwide(Collaborators, 2020); in the UK, the prevalence has increased from 390 to 570 per 100,000 population between 2000 and 2017 (King *et al.*, 2020) Biologic medications have improved the care of patients with CD. Biologic medications have been made using living organisms to target parts of the immune system and the first biologics to be introduced were anti-tumour necrosis factor-α (anti-TNF α) medications in 1999. Subsequently, vedolizumab was introduced in 2014 and ustekinumab was introduced in 2016. Studies support the postulation that biologic use is contributing to the reduced intestinal resection rate seen in the last 3 decades.

Genetic studies have shown over 240 genetic susceptibility loci for CD (Park and Jeen, 2019) and they encode proteins which are involved in the pathogenesis. There are also links between genes and early onset CD (Moran *et al.*, 2015) and those with young onset CD may be genetically different which may explain the severity in this group.

1.1.2 Intestinal Failure overview

The European Society for Clinical Nutrition and Metabolism (ESPEN) defines Intestinal failure [IF] as the reduction of gut function below the minimum deemed necessary to absorb enough macronutrients, water or electrolytes which necessitates supplementation with intravenous nutrients or fluids and electrolytes in order to maintain health or growth (Pironi *et al.*, 2015). There are 3 types of intestinal failure: type I occurs after abdominal surgery, with the need for short term parenteral support; type II IF occurs in metabolically unstable patients who may require parenteral

support for weeks to months; and type III IF occurs in patients who cannot maintain their nutrition via the intestinal tract and require long term parenteral support(Pironi *et al.*, 2015). The overall prevalence of Intestinal Failure in Europe is estimated to be between 5 and 20 per million (Pironi *et al.*, 2015; Puiggrós *et al.*, 2011; Pironi *et al.*, 2007; Bakker *et al.*, 1999; Smith *et al.*, 2011). Worldwide Crohn's disease falls into the top 3 causes for IF (Pironi *et al.*, 2015; Puiggrós *et al.*, 2011; Pironi *et al.*, 2007; Bakker *et al.*, 1999; Smith *et al.*, 2011), along with mesenteric ischaemia and surgical complications(Bond *et al.*, 2019). Crohn's disease accounts for 22.6% to 34% of patients requiring parenteral support (Smith *et al.*, 2011; Lloyd *et al.*, 2006) (Ugur *et al.*, 2009). IF is a devastating and extreme complication of CD. It is not clear why some CD patients develop IF, whilst others follow a milder disease course. The incidence of IF after an initial resection is reported as 0.8% after 5 years, and 6.1% after 15 years (Watanabe *et al.*, 2014). Studying the microbiota in this severe CD cohort may give us an insight into the pathogenesis.

1.1.3 Multiomics overview

There has been a large increase in the number of multiomics-related publications in the last 10 years. Most publications relate to gastrointestinal diseases however, there is also significant research in numerous other conditions for example, depression (Xu *et al.*, 2020), colon cancer, liver disease, and diabetes (Kanikarla-Marie and Jain, 2016), amongst others. The study of the metabolic profile can give insight into mechanistic pathways in physiology and disease.

The aetiology of CD is thought to be due to the interaction between the immune system, microbiota and the individual's genes. The genes which are linked to IBD are associated with immune regulation and epithelial function. There is evidence that the gut microbiota plays a significant role in the pathogenesis of IBD by triggering an altered immune response in susceptible individuals. The interplay between different aetiological factors in the pathogenesis of IBD leads to significant heterogeneity in this disease with different phenotypes and a spectrum of severity. In the last 10 years, profiling the microbiome and metabonome in Crohn's disease has been recognised as important as this can provide insight into the disease pathogenesis and also highlight potential biomarkers for monitoring to improve the assessment and management of this chronic condition.

1.1.3.1 Multiomics definitions

Multiomics describes an approach in which data obtained from different omic techniques such as 16s rRNA sequencing, metataxonomics, metagenomics, metabolomics, metabonomics,

metatranscriptomics and metaproteomics (Table 1) are analysed and combined; it can characterise the microbiome, its functionality through gene sequencing, and the metabolites it produces. The two most used techniques are metataxonomics and metagenomics.

Omics can provide insight into host – microbiota communications. Host- microbiota crosstalk is essential for maintaining gut homeostasis and health.

Table 1 Definitions of different omics techniques to characterise the microbiome and metabonome

Omics technique	**Definition**
Metataxonomics	The process used to sequence the entire microbiota by the amplification of taxonomic marker genes (Marchesi and Ravel, 2015)
Metagenomics	Shotgun metagenomics is to sequence the genes and genomes from the microbiota. The data is mapped to a reference database library. This technique also provides data on the function of the genes. The metagenome describes the total genes and genomes of the microbiota.
Metabolomics	The technique to determine the metabolic profile of a single sample. NMR and MS are used to characterise the metabolome.
Metabonomics	The quantitative measurement of the dynamic metabolic response of living systems to pathophysiological stimuli
Metatranscriptomics	The technique to sequence the collection of RNAs to provide data on the regulation and expression profiles of the microbiome.
Metaproteomics	The technique to determine the proteins of a sample from the microbiota.

1.1.3.2 Microbiota

The terms microbiome and microbiota are often used interchangeably but the microbiota refers to the total microorganisms of a specific body site(Lederberg and Mccray, 2022), whereas the microbiome refers to the total microorganisms and their genomes of the entire habitat and its environment. The microbiome consists of up to one trillion organisms including: bacteria, archaea, eukaryotes, protozoans and viruses but most studies focus on the bacteria and archaea as the techniques to characterise these are more widely understood and used. An individual has about 160 different species in the bowel, making up to 10^{13} to 10^{14} cells (Qin *et al.*, 2010) . The 4 main phyla are ***Bacteroidetes*** *eg. Bacteroides and Prevotella,* ***Firmicutes*** *eg. Clostridium and Ruminococcous,* ***Actinobacteria*** *eg. Bifidiobacterium*, ***Proteobacteria*** *eg. E.Coli*. (Frank *et al.*, 2007).

Traditionally, bacterial cell culture was the main way of profiling the microbiota, however, up to 70% of bacteria were unculturable using this method (Walker *et al.*, 2014), including obligate anaerobes, which predominate in the human the gut microbiome. Therefore, traditional culture methods missed most bacteria. In addition, the methods were tedious and time consuming. Significant advances have taken place with the introduction, in 2004, of high throughput omics techniques with next generation sequencing (NGS) which had a pivotal role in the surge in multiomics studies. NGS allows millions of DNA molecules with different yields and sequence lengths to be sequenced at once enabling much quicker and accurate characterisation of the gut microbiome, and their genes, proteins and metabolites.

The most common 16s rRNA techniques are metataxonomics and metagenomics. Metataxonomics, which has been the most used method for microbial profiling relies on sequencing the 16s gene from bacteria and archaea because this gene is conserved between species. Primers are used against the 16s gene, which is then amplified and then sequenced. Finally, sequences are matched against a reference database to determine the bacterial species. Some of the drawbacks include that sometimes different strains of the same species cannot be distinguished and metataxonomics cannot provide functional information. Shotgun metagenomics involves the sequencing all the extracted DNA. The DNA, once sequenced, are mapped into to a reference library to provide taxonomic and functional information. Another benefit is that metagenomics can provide information on the other microorganisms in the microbiome including viruses and fungi. A benefit of shotgun metagenomics is that it can more accurately determine the species and strain and it can sequence all the genes, so it also provides functional data and produces very large datasets; far

more than obtained from 16s sequencing. One limitation of shotgun metagenomics is that many genes may have unknown functions and also the reference library may have inaccuracies or missing annotations. It is also more expensive than metataxonomic sequencing.

Changes in the microbiota have downstream effects which is crucial to maintain intestinal homeostasis and to enhance the barrier integrity to pathogenic microorganisms. Different gut microbiota members exert either a protective or detrimental effect on the gut mucosal integrity either directly through binding to receptors, or indirectly through the effects of its metabolic products which have signalling or recognition properties, such as pattern recognition molecules, molecules that activate toll-like receptors and NOD-like receptors(Elia *et al.*, 2015), or through their production of antimicrobial peptides. One study (Rakoff-Nahoum *et al.*, 2004) describes how toll like receptors (TLRs) recognise microbial products and therefore mediate intestinal barrier function and homeostasis. In this study a mouse model deficient in TLR signalling leads to reduced protection of the colon against insults. From these results, the authors deduce that the microorganisms activate the TLRs and mediate protection from epithelial damage. When a pathogen enters the host. An innate immune response begins in which pattern recognition molecules on the cell membrane interact with the pathogen which becomes a host- antigen presenting cell which is activated and then releases cytokines which active an immune response.

1.1.3.3 Metabonomics

The metabonome is the measurement of the dynamic metabolic response to a biological challenge inside a living system. The metabolites detected might be products of host metabolism, microbial metabolism or as a result of host-microbiota interaction. The metabolites detected might be products of host metabolism, microbial metabolism or as a result of host-microbiota interaction. Nuclear magnetic resonance (NMR) or mass spectrometry (MS) are used for metabolic profiling. Metabonomic profiling can take place on any biofluid, for example, stool, urine, serum, or biopsy material. Standardised procedures regarding collection and storage of samples exist to limit the factors which can affect the metabolic profile, for example, the time between sample production and storage, time in storage, and temperature of sample collection and storage.

1.2 Confounders in multiomic studies

The inconsistencies in the findings of multiomic studies are due to the high throughput nature of the modern techniques and due to the gut microbiome and metabonome's sensitivity to the experimental process such as type of sample, handling, temperature and processing conditions, as

well as confounders such as diet, medication disease, age, BMI, infection, disease activity and surgical resection (Clooney *et al.*, 2020; Lapthorne *et al.*, 2013). At birth, the gut is sterile but becomes colonised within a few hours. The initial microbiota is influenced by type of feeding and delivery mode then the microbiome reaches maturity, at an age of around 3 years old and it remains largely stable from then on(Clemente *et al.*, 2012).The Flemish Gut Flora Project sampled the stool of 1106 patients and found that 69 covariates correlated significantly with microbiome variability (Falony *et al.*, 2016). A Dutch cohort of 1135 patients found that 126 factors related to disease, drugs, smoking and diet were associated with variations in the gut microbiome (Zhernakova *et al.*, 2016).

1.2.1 Type of sample

Stool, intestinal biopsy or rectal swabs can be used to determine the microbiome. A difference was observed when comparing the microbiome from intestinal biopsies specimens and from stool(Carstens *et al.*, 2018). Mucosal microbiota appear important and a study (Shogan *et al.*, 2014) observed changes in the mucosal associated microbiota of the anastomosis post-colectomy in rats, which were not seen in the faeces and that the mucosal microbiota reflected changes in the metabolic functions observed. However, faecal sampling remains the preferred method amongst microbiota studies as it is non-invasive and more practical.

1.2.2 Sample handling

The way the sample is handled prior to centrifugation can alter its quality. One study (Qin *et al.*, 2010) assessed the effect of temperature of the sample (4°C, 25 °C, 37 °C) and time delay (5-210 minutes) on the metabolic profile of serum samples from 28 patients. Even short delays to centrifugation significantly affected the metabolic profile and therefore should be minimised. A study by Gratton and colleagues (Gratton *et al.*, 2016) analysed changes in the microbiota amongst 5 individuals during different conditions of storage duration, temperature and freeze thaw cycles in order to create an optimised protocol for the collection of faecal samples to minimise degradation of samples. To preserve the sample, it is advised to collect the sample intact, and keep it at 4°C during transportation. Faecal water samples should be extracted, aliquoted and stored at less than -20°C and minimise the number of freeze-thaw samples.

1.2.3 Ethnicity

The incidence of CD is much lower in the developing world compared to in the West. When people migrate from the East to the West, their risk of developing CD, appears to increase to Western levels indicating environmental factors. A meta-analysis by (Misra *et al.*, 2018) reported that there was no difference in the incidence rate between Caucasians and South Asians. The leading theory is that moving to a Western, developed country, leads to changes in the diet and lifestyle such as smoking, and this can alter the microbiota to a less favourable one.

1.2.4 Antibiotics

Mice and human studies have shown that antibiotics can affect the microbiota temporarily. Whist oral antibiotics disturb the microbiota more, due to direct contact with intestinal contents, intravenous antibiotics can also affect the microbiota; especially antibiotics which are cleared by hepatic mechanisms, such as fluoroquinolones, macrolides, metronidazole and tetracyclines. These antibiotics are modified by the liver and then excreted into the gastrointestinal tract through bile. Whilst, most antibiotics, such as ciprofloxacin, vancomycin and metronidazole affect the microbiota for up to 2 weeks, some, such as amoxicillin showed perturbations for up to 2 months; some even take up to 6 months to recover (Table 2). Therefore, 8 weeks is widely used as a cut-off for exclusion in microbiome studies.

Table 2 Effect of antibiotics on the microbiota

Author	**Study**	**Changes in the microbiota**
Lewis (Lewis *et al.*, 2015)	Mice	Metronidazole – transient changes for 1-2 weeks.
Korpela (Korpela *et al.*, 2016)	142 children	Macrolides - reduced diversity, reduced *Actinobacteria*, reduced *Lactobacilli* long term, Increased *Bacteroides* and *Proteobacteria* long term Penicillin V – no change Amoxicillin – no change

Soldi (Soldi *et al.*, 2015)	15 patients with IBS	Rifaximin - increased bacterial diversity, *Firmicutes/Bacteroidetes* diversity and *F. prauznitzii.*
De la cochetiere (De La Cochetière *et al.*, 2005)	6 adults	Amoxicillin- shifts lasted average 30 day, >2 months in some.
Dethlefsan (Dethlefsen *et al.*, 2008)	3 adults	Ciprofloxacin- shifts lasted several weeks but some taxa failed to recover
Ubeda(Ubeda *et al.*, 2010)	Mice	Vancomycin caused outgrowth of *Enterobacteriaceae* with partial recovery at 2 weeks
Antonopoulos (Antonopoulos *et al.*, 2009)	Mice	Amoxicillin, metronidazole and bismuth. Cefoperazone – long term impact, Increased *Lachnospiraceae* and *Clostridaceae* and reduced diversity lasting 2-6 weeks.
Dethlefsen (Dethlefsen and Relman, 2011)	3 adults	Ciprofloxacin- reduced diversity and altered composition within 3-4 days. 43 taxa decreased (including *Faecalibacterium*) and 11 taxa increased including *Bacteroides* with partial recovery within 1 week.

1.2.5 Diet

1.2.5.1 The influence of the diet on the microbiota in health

Diet has been shown to alter the composition of the microbiome in multiple studies. The microbiota of individuals ingesting a rural African village diet which is high in fibre had higher *Prevotella*, less Bacteroides and higher diversity compared to individuals from Europe (De Filippo *et al.*, 2010). A western, high fat and high sugar diet is associated with reduced diversity and greater *Proteobacteria* (Hold, 2014). Diets low in carbohydrate and low in fibre were associated with reduced Roseburia and Eubacteria rectale and butyrate. In a study (Tao *et al.*, 2021), mice were fed a diet of EEN-n-3 polyunsaturated fatty acids (EEN-3PUFAs), which is known to be anti-inflammatory; there was an increase in *Barnesiella*, *Lactobacillus* and *Bacteroidetes*. There was an increase in acetate,

propionate and butyrate. This demonstrates the impact of diet on the microbiome and the metabonome. It is felt that the geographic difference in the incidence of IBD, with a higher incidence in the West compared to the East is partly due to dietary differences (Ng *et al.*, 2013; Hou, Abraham and El-Serag, 2011). O'Keefe and colleagues (O'Keefe *et al.*, 2015) performed a 2-week diet switch between Africans with a low fat, high fibre diet, and Americans with a high fat, low fibre diet. Adopting a high fibre diet resulted in a decrease in the stool secondary bile acids which upon changing to a high fat diet, resulted in an increase in SBAs. A high fat diet has been shown to increase hepatic PBA synthesis and increase faecal SBAs (Ridlon *et al.*, 2014; Ananthakrishnan *et al.*, 2013; Khalili *et al.*, 2018)

1.2.5.2 The influence of the diet on the microbiota in Crohn's disease

It is accepted that diet, via the modification of the microbiome, can alter inflammatory activity in IBD. Supporting evidence comes from studies which demonstrate good efficacy of enteral nutrition in inducing remission in children (Narula *et al.*, 2018). A western diet, which is high in fats, polyunsaturated fatty acids and meats, and low in fibre is thought to increase the risk of IBD. A study (Ananthakrishnan *et al.*, 2013) in which a semi quantitative diet questionnaire was longitudinally collected every 4 years, from 170,766 women, of whom 269 developed CD, showed that high dietary fibre intake was associated with a lower risk of CD. A literature review of 24 studies examining the effect of exclusive enteral nutrition on the composition of the microbiota in CD had variable results; 5 studies showed no change in microbial diversity, some showed a reduced diversity and some an increased diversity. Changes in the abundance of specific bacteria include a decrease in *Bacteroides fragilis*, *Bacteroidetes*, *Bacteroidaceae*, *Haemophilus*, *Veillonella*, *Bifidobacterium*, *Prevotella* and *Proteobacteria* amongst others (Svolos, Gkikas and Gerasimidis, 2021). The impact of different dietary components on IBD is summarised in Table 3.

Table 3 Impact of diet on the microbiota in Crohn's disease

Diet	**Effect on Crohn's disease**
Fat	Multiple case control studies have linked a high fat diet and a high long chain triglyceride diet to CD development (Mentella *et al.*, 2020) (Hou, Abraham and El-Serag, 2011).
Proteins	Animal protein from meat and fish has been shown to be a risk factor for CD development (Hou, Abraham and El-Serag, 2011), and is associated with relapse in CD (Jowett *et al.*, 2004). It has been speculated that

	breakdown of animal proteins in the gut produces substrates favouring the expansion of certain bacteria which are implicated in the pathogenesis of IBD.
Fibre, carbohydrates	Low fibre diets have been associated with an increase in the incidence of CD (Hou, Abraham and El-Serag, 2011). One theory is that when fibres are fermented in the colon, the bacterial diversity increases and SCFA production increases; SCFAs are anti-inflammatory (Christl *et al.*, 2022) (Segain *et al.*, 2000) and maintain intestinal integrity(Andoh, Bamba and Sasaki, 1999).
Additives	Food additives and emulsifiers are thought to increase inflammation and worsen colitis in animal models (Chassaing *et al.*, 2017).

1.2.6 Parenteral nutrition

An important confounder to consider in microbiota studies in patients with CD-IF is the effect of parenteral nutrition (PN) on the microbiota. There have been 6 animal studies examining the effect of PN versus a control oral diet on the microbiota in subjects without bowel resection (Table 4). These consistently show a decrease in *Firmicutes* and an increase in *Proteobacteria* and *Bacteroidetes* (Miyasaka *et al.*, 2013; Lavallee *et al.*, 2017; Heneghan *et al.*, 2014). The increase in *Proteobacteria* is also seen in studies of short bowel patients.

Table 4. Studies assessing the effect of parenteral nutrition on the microbiota.

Author	**Animal model**	**Parenteral nutrition**	**Findings**
Hodin (Hodin *et al.*, 2012)	Rats	PN for 14 days vs control diet	Decreased *Firmicutes* Increased *Bacteriodetes*
Miyasaka (Miyasaka *et al.*, 2013)	Mice	PN for 5 days	Decreased *Firmicutes* Increased *Bacteriodetes* and *Proteobacteria*
Heneghan (Heneghan *et al.*, 2014)	Mice	PN for 5 days	Decreased *Firmicutes* Increased *Bacteriodetes* and *Proteobacteria*

Harvey (Harvey *et al.*, 2006)	Piglets	PN for 7 days	Reduced diversity, increased *C. difficile*
Deplancke (Deplancke *et al.*, 2002)	Piglets	PN for 7 days	Increased C. perfringens
Lavallee (Lavallee *et al.*, 2017)	Piglets	PN for 14 days	Increased *Enterobacteriaceae*

Chapter 2: Risk factors for developing intestinal failure in Crohn's disease: a literature review and case- control study.

Abstract

Intestinal failure (IF) is an uncommon and extreme complication of Crohn's disease (CD) which is usually due to severe active disease, sequential resection leading to cumulative bowel loss or penetrating disease resulting in a shortened small bowel. Method: single tertiary centre retrospective case-control study comparing 139 patients with CD-IF and 100 patients with CD. Results: Risk factors for CD-IF in our study included: female gender, ileocolonic disease location, penetrating disease and non-exposure to biologic medications. The presence of perianal disease, upper gastrointestinal disease and extra-intestinal manifestations were not risk factors for the development of IF. Conclusions: patients with multiple risk factors for IF should have closer monitoring and have a timely escalation of treatment to avoid under treatment to reduce the chance of developing IF.

2.1 Introduction

Crohn's disease (CD) is a relapsing and remitting inflammatory bowel disease. Intestinal failure [IF] is defines Intestinal failure [IF] as the reduction of gut function below the minimum deemed necessary to absorb enough macronutrients, water or electrolytes which necessitates supplementation with intravenous nutrients or fluids and electrolytes in order to maintain health or growth (Pironi *et al.*, 2015). Rarely, CD patients develop IF, whilst others follow a milder course of their disease. Worldwide, Crohn's disease falls into the top 3 causes for IF (Pironi *et al.*, 2015; Puiggrós *et al.*, 2011; Pironi *et al.*, 2007; Bakker *et al.*, 1999; Smith *et al.*, 2011), and CD accounts for around 22.6% to 34% of patients requiring parenteral support (Smith *et al.*, 2011; Lloyd *et al.*, 2006) (Ugur *et al.*, 2009). Patients may be expected to require parenteral nutrition when the small bowel length is approximately less than 100cm without any colon in continuity, or less than 50cm with colon in continuity, however there are other important factors, such as the presence of adhesions or active disease, which means that IF can develop at much longer bowel lengths.

Patients with CD undergo intestinal resection for active disease, strictures or penetrating disease. The operation can involve formation of a stoma, such as a colostomy or enterostomy (jejunostomy or ileostomy) or can involve anastomosis between two parts of bowel.

2.1.1 Risk factors for severe Crohn's disease

In the published literature, predictors for severe CD include**: younger age at onset (<40 years), perianal disease, stricturing disease, upper gastrointestinal involvement, smoking and need for corticosteroids at first flare** (Yarur *et al.*, 2011).

2.1.2 Risk factors for post-operative recurrence

Post-operative recurrence can be categorised as clinical recurrence, endoscopic recurrence or surgical recurrence, which is the need for a second intestinal resection. Clinical recurrence is not a good predictor of endoscopic recurrence (Rutgeerts *et al.*, 1990b). Endoscopic recurrence (Rutgeert's score ≥i2) after an initial resection occurs in 34% - 59.8% at 6-12 months (de Barcelos *et al.*, 2017; Auzolle *et al.*, 2018; Thin *et al.*, 2021). Surgical recurrence after a first resection, occurs in 24.2%-28% at 5 years (Bernell, Lapidus and Hellers, 2000; Frolkis *et al.*, 2014) (Aze *et al.*, 2020)and 35%-36% at 10 years .

2.1.2.1 Literature review on post-operative recurrence

A literature review examining the risk factors for recurrence after surgical resection was performed using the search terms ((((Crohn's [Title]) AND (risk[Title])) OR (predict*[Title])) AND (recurrence[Title]) (July 2021) returned 722 items, of which 18 were included; a further 4 studies were identified from other sources.

Factors which were associated with a risk of recurrence after a surgical resection include:

- **Perianal disease**(Kim *et al.*, 2020; Bernell, Lapidus and Hellers, 2000; Khoshkish *et al.*, 2012; Chen *et al.*, 2019) (Cai *et al.*, 2019; Yang *et al.*, 2017; Gao *et al.*, 2012).
- **Microscopic inflammation** at resection margin
- **Smoking**(Clooney *et al.*, 2020; Araki *et al.*, 2018; Khoshkish *et al.*, 2012; Cottone *et al.*, 1994; Bolckmans *et al.*, 2020; Sutherland *et al.*, 1990; Kwan *et al.*, 2017) (Unkart *et al.*, 2008; Zhou *et al.*, 2018; Auzolle *et al.*, 2018) (Papay *et al.*, 2010)
- **Penetrating disease** (Chen *et al.*, 2019; Riss *et al.*, 2013; Khoshkish *et al.*, 2012; Rutgeerts *et al.*, 1990a; Lee *et al.*, 2012)
- **Lack of TNF agent post-operatively** (Kusaka *et al.*, 2017; Auzolle *et al.*, 2018)
- **Lack of immunomodulators post-operatively** (Unkart *et al.*, 2008; Papay *et al.*, 2010)

Factors which were not consistently associated with the risk of recurrence included: age at diagnosis (de Barcelos *et al.*, 2017; Navaratne *et al.*, 2021; Caprilli *et al.*, 1996; Chen *et al.*, 2022; Cai *et al.*,

2019), disease duration (Caprilli *et al.*, 1996; Wettergren and Christiansen, 1991; de Barcelos *et al.*, 2017), male gender (Unkart *et al.*, 2008; Wettergren and Christiansen, 1991; de Barcelos *et al.*, 2017), and family history (Unkart *et al.*, 2008).

2.1.3 Risk factors for intestinal failure

There are only a few studies examining the risk factors for the development of IF in CD. One study of 82 patients with CD-IF (Gearry *et al.*, 2013b) found that the factors associated with the development of IF were: **younger age at diagnosis** (<16 years), **stricturing disease**, **younger age at first surgery** and **family history** of inflammatory bowel disease (IBD). Another study of 21 patients with CD-IF (Watanabe *et al.*, 2019) found that **non-use of anti-TNF therapy** was an independent risk factor for the development of IF, however, numbers were small with 21 IF patients.

2.2 Aims

The aim of this study was to identify risk factors for developing IF in patients with CD-IF compared to a control group of CD (without IF).

2.3 Hypothesis

We hypothesise, that risk factors for IF in CD will be the same as the risk factors for post-operative recurrence, such as, younger age at diagnosis, smoking, the presence perianal disease and penetrating disease. We hypothesise that patients with non-exposure to adequate trials of biologic medications are more likely to develop IF.

2.4 Methods

A single tertiary centre case-control retrospective study of 139 patients diagnosed with CD-IF between 1980 and 2018 and 100 controls with CD without IF were selected from consecutive IF and IBD clinics respectively. CD patients (without IF) were selected from the local geographic area, therefore, excluding tertiary referrals for complex Crohn's disease.

Inclusion criteria: >18 years, patients with CD-IF due to small bowel Crohn's disease, receiving home parenteral nutrition (HPN) or home intravenous fluids and electrolytes (HIVF) for over 1 year (type III IF).

Exclusion criteria: patients with colonic CD.

Variables analysed are shown in Table 6. Family history and smoking were not analysed as these data were often missing from the records. Patients were regarded as having perianal disease if they had perianal fistulae, fissures, abscesses, or collections at any point during their life. A patient was regarded as having had an adequate exposure to a biologic medication (anti-TNF, vedolizumab or ustekinumab) if: they were still on a biologic, if a biologic was discontinued for non-response or loss of response, or if a biologic had been stopped due to remission. Patients were categorised as having non-exposure to biologics if: they were non-compliant, it they had an adverse event or intolerance within the first 6 weeks resulting in discontinuation, if it was not available, or if it was never tried. If a patient had received more than 1 biologic, they were regarded as being exposed if they had an adequate exposure to at least one biologic.

Continuous data were presented as mean (+/- standard deviation) or median (interquartile range) and categorical data were presented as a proportion. Baseline differences were compared using the chi-squared test for categorical data and the T-test for parametric continuous data and the Mann-Whitney U test for non-parametric data. Univariate logistical regression was used for variables. Analysis was conducting using SPSS Version 27. A result was considered significant if $p<0.05$.

2.5 Results

139 patients with CD-IF and 100 patients with CD (without IF) were included.

2.5.1 Clinical features of the Crohn's Disease – intestinal failure group

139 patients with CD-IF had a median age of 57.5 years at the time of their most recent clinic appointment and 64% were female (Table 6). The median age at diagnosis of CD was 21 years. The median time from diagnosis of CD to diagnosis of IF was 20.5 years. The mean small bowel length (BL) was 107.6cm; mean bowel length (BL) 107.0 cm for females and 108.7 cm for males. There was no difference in the mean bowel length between patients receiving HPN (n=107) and HIVF (n=32) with mean small bowel lengths of 105cm and 113cm respectively (p=0.87). 123 patients had previous small bowel resections (SBRs) and colonic resections, 14 patients had only SBRs, and 2 patients had never had any bowel resections but had extensive active small bowel inflammation causing weight loss and obstructive symptoms (Table 5). Patients were regarded as having had both SBR and colonic resection if they had a previous ileocolonic resection or right hemicolectomy or a SBR and segmental colectomy. 38/139 had colon in continuity (CIC); 35 had CIC via a surgical anastomosis and 3 had the entire colon in continuity with an intact ileocaecal valve. 101 patients had an end enterostomy (Table 6). Of those with CIC: 3 patients had <50% of their colon in

continuity, 31 had >50% of their colon in continuity, 1 had an ileorectal anastomosis and 3 had the entire colon in situ.

43 patients had perianal disease and 11 patients had upper gastrointestinal disease involvement. Montreal location at diagnosis was: 27 ileal disease (L1), 7 L2 disease (colonic) and 91 ileocolonic disease (L3). At follow-up, 25 had L1 disease and 111 had L3 disease, indicating that the extent of the inflammation had progressed in 9 patients. Montreal behaviour at diagnosis was: 46 inflammatory disease (B1), 34 stricturing disease (B2), 50 penetrating disease (B3) and data were missing for 11 patients. At last follow- up: 34 had B1 disease, 29 B2 disease and 69 had B3 disease, indicating that the disease had progressed in 17 patients.

The predominant cause of IF was active disease in 126 patients and surgical complications in 13 patients; 4 had bowel ischaemia post operatively, 3 had a leak, 1 had multiple stoma refashions due to mechanical issues and 5 had adhesions.

30/139 patients had previously received biologic medications prior to the development of IF; 2 were still on it, 25 had experienced a non-response or loss of response, and 3 had an adverse reaction. 109 had not previously received biologic medications; the main reason was because the patients last had active disease prior to the introduction of biologics in 1999 or may not have tried it due to contraindications or may have proceeded to surgery. Overall, 27/139 (19.4%) were regarded as having had an adequate exposure to biologics prior to the development of IF. A further 18 patients received biologics after the development of IF.

2.5.2 Clinical features of the Crohn's Disease (without IF) group

Patients with CD had a median age of 40 years at the time of their latest clinic appointment, with a median age at diagnosis of CD of 23 years and 45% were female (Table 6). The small bowel length was unknown. 4 patients had only had small bowel resections (SBRs) and 40 patients had SBR and colonic resections and 56 patients had never had an operation (Table 5). Of the 44 patients who had operations; 36 had an anastomosis and 8 had an enterostomy. 65 had an intact ileocaecal valve of which 55 had no prior surgeries and 10 had SBRs and segmental colectomy only. Montreal location at diagnosis was: 42 had L1 disease, 10 had L2 disease and 42 had L3 disease. At follow-up, 36 had L1 disease, 0 had L2 disease and 58 had L3 disease indicating progression of disease extent in 16 patients. Montreal behaviour at diagnosis was: 74 had B1 disease, 18 had B2 disease and 6 had B3

disease. At follow-up, 65 had B1 disease, 24 had B2 disease and 11 had B3 disease indicating progression in 14 patients.

2.5.3 Differences between CD-IF and CD (without IF)

CD-IF patients were older, had a longer duration of Crohn's disease until the clinic, more often had an enterostomy (74% vs 8%), were less likely to have current use of biologic medications (8.6% vs 49%) at the latest clinic and were twice as likely to be in remission in the last year (75.5% vs 37%). There was no difference in the average CRP (Table 6). 98.6% of CD-IF patients had had at least 1 small bowel resection compared to 44% of CD patients. The average number of small bowel resections were 2.9 and 0.6 respectively. There was at least one surgical anastomosis in 35/139 (25.1%) of CD-IF patients compared to 36% of CD patients. Progression to L3 disease occurred equally in both groups (9/125 CD-IF vs 16/94 CD, p=0.077).

In the CD-IF group, only 48/139 had been prescribed biologic medications prior to the latest clinic; of these only 30 had tried biologic medications prior to their diagnosis of IF. 27/139 were deemed to have had an adequate trial of biologics prior to the diagnosis of IF, 3 patients had adverse reactions to 1 or more biologics which meant they did not get adequate exposure. In the CD group, 65/100 had been prescribed at least one biologic previously; of which 59 were deemed to have had adequate exposure and 6 had termination due to an adverse event.
Out of 85 biologic encounters in the CD-IF group, there were 25 adverse events (29.4%).
There were far fewer adverse events in the CD group with in with 12 adverse events in 110 biologic encounters (10.9%) (Table 6).

The was no difference in the CRP between CD-IF and CD patients. CD-IF patients often have concurrent infections which may explain some of the lack of a difference, however if patients had a documented infection, that CRP was not counted and the previous CRP was used. A raised CRP >10 mg/dl was observed in 14/105 patients with CD-IF in remission; they may have had active perianal disease, a recent infection or active arthropathy. 7 of 34 with active disease had a CRP $\leq$10 mg/dl; this shows that CRP may not always rise with active disease; even patients with a few ileal ulcers were regarded as having active disease in this study. The average CRP amongst those with active disease was 9.33 mg/dl compared to 5.46 in the remission group (p=0.09) but this was not significant.

2.5.3 Risk factors for CD-IF

Female gender (OR 2.25 P=0.002), ileocolonic disease at diagnosis (p<0.001), ileocolonic disease at last follow up (p=0.002), penetrating disease at diagnosis (UVA p<0.001), penetrating disease at last follow up (p<0.001) and non-exposure to biologic medications (OR 6.75, p<0.001) (Table 6) were significant risk factors for IF. Non-exposure to biologic medication remained a significant risk factor for IF, even when disease activity was added as a covariate (OR 4.09, P<0.001)

Factors which were not significantly associated with IF in our study included: age at diagnosis of CD <40 years, age at first surgery, perianal disease, upper gastrointestinal (UGI) disease and extra-intestinal manifestations (Table 6).

Table 5 Types of surgery comparing CD-IF and CD (without IF)

SBR, small bowel resection.

Resections	**CD (n=100)**	**CD-IF (n=139)**
Colonic resection and SBR	40	123
SBR only	4	14
No operations	56	2

Table 6 Comparing clinical features in CD-IF versus CD (without IF)

Categorical data were compared using the chi squared test. For continuous data, the Mann-Whitney U test[#] was used for non-parametric data. Univariate logical regression was used for variables.[+]

CI, confidence interval; IF, intestinal failure; LOR, loss of response; NR, non-response; OR, odds ratio.

Total	**CD (n=100)**	**CD-IF (n=139)**	***P* value**
Age median (IQR), y	40 (29.0, 57.5)	57.5 (48.6, 66.7)	**<0.001**[#]
Gender n	45F 55M	89F 50M	**OR 2.25 95% CI (1.3-3.8), p 0.002** [+]
Age at diagnosis of Crohn's disease median (IQR), y	23 (19.0, 31.0)	21 (16.0, 30.0)	0.07 [#]

Age at diagnosis of Crohn's disease	<40y 83 ≥40y 17 <17 18 ≥17 82	<40y 122 ≥40y 17 <17 38 ≥17 101	0.29 0.09
Duration of Crohn's disease median (IQR), y	12(7.5, 21.5)	33 (24, 42)	**<0.001** #
Duration of Crohn's disease to IF median (IQR), y	n/a	20.5 (11.3-30)	
Age at first surgery median (IQR), y	26.5 (20.0, 39.3)	26 (26.0, 37.8)	0.99
Number of small bowel resections	0-56 1-32 2-9 3-2 4-1	0-2 1-19 2-43 3-38 4-14 5-16 6-4 7-3	
No of colonic resections	0-61 1-33 2-4 3-2	0 – 14 1 – 72 2- 38 3 - 12 4 – 1	
Length of remaining colon in continuity	End enterostomy 8 IRA 1 >50% 31 Entire colon 4 No surgery 56	End enterostomy 101 IRA 1 <50% 3 >50% 31 Entire colon 1 Normal (no surgery) 2	0.12
Number of anastomoses	None- 64	None - 103	0.07

	≥1-36	≥1 – 36	
Enterostomy **Colon in continuity (CIC)**	Enterostomy 8 CIC 92	Enterostomy 105 CIC 34	**0.001**
Perianal disease	Yes 33 No 67	Yes 43 No 96	0.75
Upper gastrointestinal disease	Yes 3 No 97	Yes 11 No 128	0.11
Extra-intestinal manifestations	Yes 6 No 94	Yes 13 No 126	0.37
Location at diagnosis	L1 42 L2 10 L3 42 6 missing data	L1 27 L2 7 L3 91 14 missing data	**<0.001**
Location at last follow up	L1 36 L2 0 L3 58 6 missing data	L1 25 L2 0 L3 111 3 missing data	**0.002**
Behaviour at diagnosis	B1 74 B2 18 B3 6 2 missing data	B1 46 B2 34 B3 50 9 missing	**<0.001**
Behaviour at last follow up	B1 65 B2 24 B3 11	B1 34 B2 29 B3 69 7 missing	**<0.001**
Biologic adequate exposure prior to IF diagnosis in CD-IF and prior to last clinic in CD	**Exposed 59** 4 remission 49 still on it 6 NR **Not exposed 41** 6 adverse events 28 not tried or not indicated. 7 active prebiologic era **Exposed 59/100=59%**	**Exposed 27** 2 still on it 25 NR/LOR **Not exposed 112** 3 adverse events 109 not tried or active prior to the biologic era **Exposed 27/139= 19.4%**	**OR 6.75** **95% CI (3.8 – 12.0)** **(p<0.001)** [+]
Biologic history prior to last clinic	**Yes received 65** 4 remission 49 still on it 6 NR/LOR 6 adverse **Not received 35**	**Yes received 48** 12 still on it 29 NR/LOR 7 adverse **Not received 92**	

	28 not tried or not needed 7 active pre biologic era	45 not tried 47 active pre biologic era	
Proportion of patients who have experienced an adverse effect to at least one biologic	Out of 110 biologics episodes in 65 patients **<u>12 adverse effects in 10 patients</u>** 1 cancer 2 intolerant 3 reaction 6 rash **Overall 10/65 =15.4% patients had an adverse event**	Out of 85 biologics episodes in 48 patients **<u>25 adverse events in 22 patients</u>** 4 joint pains 4 intolerant 9 anaphylaxis/reaction 3 rash 1 TB 4 infection **Overall 22/48= 45.8% patients had an adverse event.**	
C-reactive protein mean ± SD (mg/dl)	**6.46 ± 2.5**	**6.43 ± 2.6**	0.99 #
Biologic use at recent clinic visit	Yes 49 No 51	Yes 12 No 127	**0.0001**
Remission last 1 year	Yes 37 No 63	Yes 105 No 34	**<0.001**
Remission last 3 years	Yes 24 No 75	Yes 98 No 39	**<0.001**
Remission last 5 years	Yes 23 No 73	Yes 90 No 46	**<0.001**

2.6 Discussion

In this case-control study, I compared patients with CD-IF who had been referred from a large geographic area to patients with Crohn's disease from the local geographic area who did not have IF; this introduces bias as clinical features may differ geographically. Ideally, control patients would have been selected from a wide geographical area, however, we did not have access to patient records from other hospitals. In our study, CD-IF patients were older, which reflects IF patients having been referred from other areas of the UK with likely a longer disease course compared to a local CD cohort. More patients had a stoma in the CD-IF group which represents the disease course with multiple operations which often result in a stoma due to loss of the colon from active disease,

loss of the rectum due to perianal disease, or due to the need for a stoma due to complications. More patients with CD-IF were in remission compared to those with CD. A study (Gearry *et al.*, 2013b) from the same institution but with only 2 patients in common due to a lack of access to records, found that family history of IBD and age of CD diagnosis < 16 years were associated with the development of IF. We did not find that age <17 years at diagnosis of CD was a risk factor for IF. We did not assess family history or smoking, due to missing data.

Risk factors for IF in CD that we identified in our study included female gender which was expected since females have a shorter starting small bowel length (Bekheit *et al.*, 2020). Ileocolonic disease, at diagnosis and follow up, as expected, was a risk factor for IF; patients with ileocolonic disease may have a more aggressive disease, or may be more likely to require colectomy and therefore need a longer small bowel length to avoid IF. Whilst progression to L3 disease was similar in both groups, due to the challenges of patients being referred from elsewhere and therefore a lack of detailed notes, the time to progression was not compared.

2.6.3 Penetrating disease*:* CD-IF patients more often had penetrating disease at diagnosis (38% CD-IF vs 6% CD) and at follow-up (52% CD-IF vs 11.0% CD). Penetrating disease is associated with a more aggressive disease course and more often requires surgery. In some cases, it was not clear whether an ECF was post-operative or due to Crohn's disease inflammation. In these cases, histology, endoscopy and time after surgery was determined. Those who developed ECF's due to active Crohn's disease were counted as having penetrating disease.

2.6.4 Non-exposure to biologics*:* CD-IF patients were less likely to have had an adequate trial of biologics prior to the development of IF (19.4% vs 59%). Between 1955 and 1998 (pre-biologics era), within 10 years of diagnosis, the risk of requiring bowel resection surgery was 40-71% (Bouguen and Peyrin-Biroulet, 2011; Bernell, Lapidus and Hellers, 2000), and it decreased to 25-33% (Bouguen and Peyrin-Biroulet, 2011) between 1998-2005. The risk of second resection within 10 years has also fallen from 44.6% prior to 1980, to 33.2% post 1980 (Frolkis *et al.*, 2014). As expected, the incidence of IF in CD has also fallen in the last 3 decades (Oke and Gabe, 2017; Smith *et al.*, 2011; Ugur *et al.*, 2009), in parallel to the reduction in resection rates. Data from home parenteral support registries show that the proportion patients who have IF due IBD, has fallen from 37.9% (pre-1989) to 22.6% (2010-2016) ($p<0.001$) (Oke and Gabe, 2017; Ugur *et al.*, 2009). The single biggest factor for the

reduction in surgical resection rates and IF in CD is thought to be the introduction of, and the less restricted use of medical treatments for CD.

Thiopurines have been used for 50 years and biologics are medications are newer. Anti-tumour necrosis factor-α (anti-TNF α) medications were introduced in 1999. Subsequently, vedolizumab was introduced in 2014 and ustekinumab was introduced in 2016. Studies support the postulation that biologic use is contributing to the reduced intestinal resection rate seen in the last 3 decades. In the ACCENT I trial, 3% of patients receiving Infliximab had surgery by week 54, compared to 7.5% of those receiving on-demand treatment (p=0.01) (Hanauer *et al.*, 2002). Similarly, in the CHARM trial, 0.6% of patients receiving Adalimumab had surgery by 1 year compared to 3.8% in the placebo group (p=0.001) (Colombel *et al.*, 2007). In our study, only 19.4% of CD-IF patients had been adequately exposed to biologics prior to the development of IF compared to 59% in the CD group (Table 6). Reasons for non-exposure in the CD-IF group were: 3 adverse events, 109 either had active disease prior to the availability of biologics or were not tried for unclear reasons which may include contraindications or surgery being chosen. However, given the aggressive disease course, it is thought that the majority of the 139 patients with IF due to active disease may have benefitted from biologics, had they been available. The reasons for non-exposure in the CD group were 7 had active disease prior to biologic availability, 6 had adverse events and the reasons are not clear for 28 patients, however in this group, it may have not been indicated due to lack of moderate to severe disease.

Despite other studies showing that young age at CD diagnosis was a risk factors for more severe disease (Yarur *et al.*, 2011) and a risk factor for IF (Gearry *et al.*, 2013a), in our study there was no significant association. Young age at diagnosis of CD was not associated with risk of post operative recurrence of CD in multiple studies (Caprilli *et al.*, 1996; de Barcelos *et al.*, 2017; Navaratne *et al.*, 2021; Chen *et al.*, 2019). In our study, out 38 patients had CD onset < 17 years, of whom 6 patients were diagnosed with CD age 10 or less.

Age at first surgery was not found to be a risk factor for CD-IF compared to CD (without IF) p=0.99. **The findings of this study support the findings in the literature. Planned work includes increasing the number of control patients to increase the power of the study. There is a surplus of control patients without intestinal failure, and increasing numbers should be attainable.**

2.7 Conclusion

Predictors of developing IF in Crohn's disease include penetrating disease at diagnosis and follow-up, female gender, non-exposure to biologic medications and ileocolonic disease location at diagnosis and at follow up. Patients with these risk factors should have closer monitoring and have a timely escalation of treatment to avoid under treatment to reduce the chance of developing IF.

Perianal disease, upper GI disease, extra intestinal manifestations (EIMs), young at diagnosis or age at first surgery were not risk factors for CD-IF in our cohort, despite being shown to be a risk factor for CD recurrence or severe CD in other studies.

Chapter 3: Patients with Crohn's disease with intestinal failure are more often in remission compared to Crohn's disease without intestinal failure: a case-control study.

Abstract

Intestinal failure (IF) is an uncommon and extreme complication of Crohn's disease (CD). Our single tertiary centre experience is that patients with CD-IF are often in remission and the factors responsible for this are not clear. Methods: Retrospective case control study comparing 139 patients with CD-IF and 100 patients with CD (without IF). Results: The rate of 1-year remission in the CD-IF group was double that of the CD group (75.5% vs 37%). Only 8.6% of patients in the CD-IF group were currently receiving biologic medications compared to 48% in the CD group. This shows that Crohn's disease activity appears to burn out after IF develops. Comparison between CD-IF patients with active disease vs remission found that the strongest risk factor for active disease in the CD-IF cohort was the presence of a surgical anastomosis. The presence of UGI disease and penetrating disease were also risk factors for active disease. The presence of perianal disease and EIMs were not risk factors for active disease. Although CD-IF patients did not have very active disease, patients with CD-IF should still be regularly monitored for active disease, especially if they have an anastomosis, UGI disease or penetrating disease.

3.1 Introduction

Intestinal failure (IF) is an uncommon complication of severe Crohn's disease (CD) due to extensive or recurrent small bowel resections or operative complications. It has been observed that patients with CD-IF often enter remission after the development of IF, as the disease appears to 'burn out'. The rate of remission in the CD-IF group is high and very few patients require biologic medications. The factors responsible for the high remission rate in CD-IF are not clear but could include insufficient bowel length to inflame, changes in the microbiota or metabonomic profile due to surgery and increased oxygen and bile content of the small bowel, dietary changes, or parenteral nutrition, or due to a high proportion of patients having an enterostomy (jejunostomy or ileostomy) which avoids an anastomosis, which is a risk factor for inflammation. Identifying risk factors for active disease in patients with CD-IF can help to risk stratify and counsel patients and guide treatment options.

In the published literature, it is difficult to estimate the proportion of CD-IF that is due to operative complications, due to the heterogeneity in classification of causes of IF in different studies. However,

in one study of 121 patients (Soop *et al.*, 2020) , the cause of IF was felt to be post operative sepsis related to an anastomosis in 32/121 (26%) of patients. In our cohort, in approximately 13/139 (9.4%), the predominant reason for IF was felt to be surgical complications: 3 had a leak or post-operative ECFs, 5 had adhesions (bowel length (BL) 80-200cm), 4 had ischaemia (BL 15cm-120cm), and 1 patient had multiple stoma refashions due to mechanical issues; assignment of aetiology was performed by the author and blinded observers was not undertaken.

Patients with IF due a surgical complication may be expected to have less active disease than those with IF due to sequential active disease, however, risk factors for active disease have been analysed on the total cohort of CD-IF patients irrespective of aetiology. During intestinal resection surgery, the decision to perform an end enterostomy rather than perform an anastomosis (either entero-enteric or entero-colic) depends on many factors including patient choice, length of remaining small bowel and length of colon, presence of disease activity and surgical factors.

3.2 Aim

To compare the proportion of patients in remission (1 year, 3 years and 5 years) in patients with CD–IF to CD (without IF) and examine risk factors for active disease in patients with CD-IF.

3.3 Methods

A single, tertiary centre, retrospective study. Patients with CD-IF diagnosed between 1980 and 2018 were selected from a prospectively maintained IF database. Control patients with small bowel CD without IF who lived in the local geographic area were selected from consecutive Gastroenterology clinics. Remission was defined as an absence of any inflammation on endoscopic and/or radiological examination, with a faecal calprotectin <100 μg/g and the absence of acute therapies for CD in the past 1, 3 and 5 years prior their latest clinic appointment. The simple endoscopic score for Crohn's disease (SES-CD) and the Crohn's disease endoscopic index of severity scores (CDEIS) were not used as our patients have had had surgical resections. Crohn's Disease Activity Index score (CDAI) and Harvey Bradshaw Index (HBI) $\leq$4 was not used as the majority of patients with IF have a stoma so cannot report stool frequency, and they may have other reasons for the abdominal pain or reduced general well-being.

Only CRP results obtained from an outpatient setting were used, and if an infection was present, the previous CRP was used. Only the most recent nutrition support type documented, as some patients were previously on IVF and then HPN, or vice versa.

Categorical data were compared using the chi squared test. For continuous non-parametric data, the Mann-Whitney U test was used, for parametric data, the T test was used. Univariate logistic regression was used for variables. A p value of <0.05 was considered significant.

3.4 Results

3.4.1 Patient characteristics

139 patients with CD-IF were included with a median age of 57.5 years at the time of their most recent clinic appointment and 64% were female. The median age at diagnosis of CD was 21 years, the median duration from CD to the development of IF was 20.5 years (IQR 11.3, 30.0). The mean small bowel length (BL) was 106.1cm; mean BL was 92.3cm for patients with colon in continuity (n=38) and 103.4cm for patients with an end enterostomy (n=101). 97 patients had ongoing IF, 18 died, 1 received teduglutide, 1 received an intestinal transplant and 21 patients had achieved nutritional autonomy after a range of 2-11 years (mean 7.1 years). 137/139 patient had previous intestinal resection.

100 patients with CD (without IF) had a median age of 40 years at the time of their latest clinic appointment, with a median age at diagnosis of CD of 23 years and 45% were female. 44/100 patients had previous intestinal resections.

3.4.2 Comparing remission rates in Crohn's disease-intestinal failure vs Crohn's disease.

More patients were in remission for 1 year in the CD-IF group compared to the CD (without IF) group (75.5% and 37% respectively, p<0.001) (Table 7).
3 - year remission rates were also higher in the CD-IF group (71.2% vs 24.2%, p<0.001).
5-year remission rates were also higher in the CD-IF group (66.2% vs 24.0%, p<0.001) (Table 7).
Baseline differences included longer small bowel length for those with active disease, but this was not significant.

The CRP was lower in those with remission than active disease in the 1 year (Table 8) and 5- year remission groups (Table 10). Patients in the 5- year remission group had CD for longer and had a longer duration between CD and IF. There was no difference in the age of patients in both groups, nor the age at diagnosis of CD, age at diagnosis of IF, duration of IF, or duration of CD.

3.4.3 Risk factors for active disease

105 patients in remission had a mean small bowel length (BL) of 103cm were compared to 34 patients with active disease with a mean small BL of 120cm.

Risk factors for active disease for the previous 1, 3 and 5 years (Table 8-10) were the presence of a surgical anastomosis with the colon, and upper gastrointestinal (UGI) disease involvement. Absence of penetrating disease behaviour was associated with 5- year remission. Shorter duration between CD diagnosis and IF diagnosis was associated with active disease in the 3- and 5 year groups.

Factors which were not associated with active disease were the presence of perianal disease, EIMs, number of small bowel resections, disease location or behaviour at diagnosis or follow up, however, absence of penetrating disease at follow up is associated with 5 year-remission (Table 10).

Table 7.Comparing the remission rates between CD-IF and CD (without IF)

	CD (100)	**CD-IF (139)**	
Remission 1 year	37/100 (37%)	105/139 (75.5%)	**<0.001**
Remission 3 years	24/99 (24.2%)	98/137 (71.5%)	**<0.001**
Remission 5 years	23/96 (24.0%)	90/136 (66.2%)	**<0.001**

Table 8. Risk factors for active disease (past 1 year) in patients with CD-IF

	Remission (105)	**Active (34)**	P value
Age, median (IQR), y	60.2 (50.2, 67.2)	53.5 (43.7, 63.5)	0.06
Gender	68F 37M	21 F 13M	0.75
Age at diagnosis of Crohn's disease, median (IQR), y	21 (16.0, 33.0)	21 (16.5, 25.0)	0.65

Duration of Crohn's disease median (IQR), y	34 (24.0, 42.0)	30 (18.5, 21.0)	0.14
Duration of CD until IF, median (IQR), y	22.5 (12.8, 30.3)	20 (9.25, 29.0)	0.40
Age at diagnosis of IF, median (IQR), y	46 (39.8, 57.0)	46 (32.0, 56.5)	0.35
Bowel length (mean± SD), cm	102.8 ± 43.0	120.0 ± 55.0	0.09
Parenteral nutrition (PN) or fluids (HIVF)	PN 80 HIVF 25	PN 27 HIVF 7	0.69
Enterostomy vs continuity with colon	CIC 19 Enterostomy 86	CIC 18 Enterostomy 16	**OR 4.3 (95% CI 1.8-10.0) p=<0.001**
Number of small bowel resections mean (range)	3.00 (1-7)	2.49 (0-6)	0.08
Number of small bowel resections	1 – 13 2 - 32 3- 31 4- 10 5- 13 6- 3 7- 3	0-2 1-6 2-11 3-7 4-4 5-3 6-1	
Perianal disease	Yes 32 No 73	Yes 11 No 23	0.84
Upper gastrointestinal disease	Yes 5 No 100	Yes 6 No 28	**0.01**
Extra-intestinal manifestations	Yes 9 No 96	Yes 4 No 30	0.58
Disease location at diagnosis	L1 21 L2 7 L3 64 Missing data 13	L1 6 L2 0 L3 27 Missing data 1	0.25
Disease location at follow up	L1 20 L2 0 L3 82 Missing data 3	L1 5 L2 0 L3 29	0.49

Disease Behaviour at diagnosis	B1 39 B2 25 B3 34 Missing data 7	B1 7 B2 9 B3 16	0.15
Disease Behaviour at follow up	B1 28 B2 20 B3 51 Missing data 6	B1 6 B2 9 B3 18	0.24
C-reactive protein mean ± SD, (mg/dl)	5.3	9.8	**P=0.04**
Currently receiving biologics	No 101 Yes 4	No 28 Yes 6	**0.007**

Table 9. Risk factors for active disease (past 3 years) in patients with CD-IF

	Remission (98)	**Active (39)**	P value
Age (mean± SD), y	58.6 ± 12.6	52.5 ± 13.6	0.08
Gender	65F 33M	23F 16M	0.42
Age at CD diagnosis (mean± SD), y	24.6 ± 12.0	23.1 ± 12.0	0.50
Duration of CD (mean± SD), y	33.9 ± 12.6	29.2 ± 15.3	0.07
Duration of CD until IF (mean± SD), y	23.3±13.3	20.1±13.7	0.12
Age at diagnosis of IF (mean± SD), y	47.9±14.2	43.2±14.1	0.10
Duration of IF (mean± SD), y	10.6 ± 6.8	9.1±8.1	0.27
Bowel length (mean± SD), cm	103 ± 42.7	119 ± 55.3	0.07
Parenteral nutrition (PN) or fluids (HIVF)	PN 76 HIVF 22	PN 31 HIVF 8	0.80
Enterostomy vs Colon in continuity	CIC 15 Enterostomy 83	CIC 17 Enterostomy 22	**OR 3.8 (95% CI 2.4-6.0) <0.001**
Number of small bowel resections mean (range)	2.98 (1-7)	2.68 (0-7)	0.29

Number of small bowel resections	1 – 12 2 – 31 3- 31 4- 9 5- 10 6- 3 7- 2	0-2 1-7 2-12 3-7 4-4 5-5 6-1 7-1	
Perianal disease	Yes 30 No 68	Yes 12 No 27	0.97
Upper gastrointestinal disease	Yes 4 No 94	Yes 7 No 32	**0.007**
Extra-intestinal manifestations	Yes 9 No 89	Yes 4 No 35	0.813
Disease location at diagnosis	L1 18 L2 7 L3 62 Missing data 11	L1 8 L2 0 L3 28 Missing data 3	0.21
Disease location at follow up	L1 17 L2 0 L3 78 Missing data 3	L1 7 L2 0 L3 32 Missing data 0	0.67
Disease Behaviour at diagnosis	B1 38 B2 21 B3 33 Missing data 6	B1 7 B2 13 B3 16 Missing data 3	0.11
Disease Behaviour at follow up	B1 28 B2 17 B3 47 Missing data 6	B1 6 B2 12 B3 20 Missing data 0	0.15
CRP (mg/dL)	5.64	8.61	0.06

Table 10. Risk factors for active disease (past 5 years) in patients with CD-IF

	Remission (90)	**Active (46)**	P value
Age (mean± SD), y	58.9 ± 12.4	52.6 ± 13.7	0.08
Gender	62F 28M	26F 20M	0.15
Age at CD diagnosis, (mean± SD), y	23.8 ± 10.9	25.2 ± 13.9	0.52

Duration of CD (mean± SD), y	35.1 ± 12.0	27.3 ± 15.0	**0.001**
Duration of CD until IF (mean± SD), y	24.2±13.0	18.4±13.3	**0.004**
Age at diagnosis of IF (mean± SD), y	48.0±14.0	43.5±14.5	0.12
Duration of IF (mean± SD), y	10.9 ± 7.0	9.0±7.8	0.14
Bowel length (mean± SD), cm	101.2 ± 43.3	120.8 ± 52.3	**0.03**
Parenteral nutrition (PN) or fluids (HIVF)	PN 71 HIVF 19	PN 36 HIVF 10	0.93
Enterostomy vs Colon in continuity	CIC 14 Enterostomy 76	CIC 18 Enterostomy 28	**OR 2.7 (95% CI 1.7-4.2) <0.001**
Number of small bowel resections mean (range)	2.92(1-7)	2.62 (0-7)	0.18
Number of small bowel resections	1 – 11 2 – 28 3- 28 4- 9 5- 10 6- 2 7- 2	0-2 1-8 2-15 3-10 4-4 5-5 6-1 7-1	
Perianal disease	Yes 28 No 62	Yes 14 No 32	0.94
Upper gastrointestinal disease	Yes 3 No 87	Yes 8 No 38	**0.004**
Extra-intestinal manifestations	Yes 8 No 82	Yes 5 No 41	0.71
Disease location at diagnosis	L1 16 L2 6 L3 58 Missing data 10	L1 10 L2 1 L3 31 Missing data 5	0.41

Disease location at follow up	L1 15 L2 0 L3 72 Missing data 3	L1 9 L2 0 L3 37 Missing data 0	0.47
Disease Behaviour at diagnosis	B1 36 B2 20 B3 29 Missing data 5	B1 9 B2 14 B3 19 Missing data 4	0.09
Disease Behaviour at follow up	B1 28 B2 15 B3 42 Missing data 5	B1 6 B2 14 B3 24 Missing data 2	**0.03**
CRP (mg/dL)	5.67	8.17	**0.04***

3.5 Discussion

It has been observed that CD activity tends to remit, and fewer medications are required once IF develops, however, little data exists on this and is not consistent. This finding is not supported by another study of 86 patients with Crohn's disease (Singh, Nan and Shen, 2022) in which the clinical activity was assessed before and 6-12 months after the diagnosis of short bowel syndrome. Patients had a mean small bowel length of 154cm, 77% had a stoma and 12.5% had an ileocaecal valve; there was no difference in the endoscopic disease activity before and after the diagnosis of SBS. However, in this study, details of the cause of the intestinal failure and the proportion who had active disease before and after is not known, but also the follow-up duration is short. Patients in our study had at least 5 years of follow up in 136 of the 139 patients. In our study, remission rates were higher in the CD-IF group compared to the CD (without IF) group (75.5% vs 37.0%, p<0.001).

We found the following risk factors for active disease: presence of an anastomosis with the colon (OR 4.3, p= <0.001, penetrating disease at follow-up for 5 year (p=0.03), and the presence of UGI disease (p=0.01). Perianal disease and EIMs were not risk factors for active disease.

3.5.1 Presence of an anastomosis

In our study, 102 patients had an end enterostomy and 35 patients had at least one surgical anastomosis and 2 patients had an intact ICV. The presence of an anastomosis was significantly associated with having active disease. 17/35 (48%) of those with a surgical anastomosis with part of the colon had active disease, compared to 16/102 (16%) of those with an enterostomy (OR 4.3, p=<0.001). The type of anastomosis has been well studied and has been regarded as an established risk factor for the recurrence of inflammation after resection. The consistent finding is that a side-to-side anastomosis confers a lower risk of post-operative recurrence of Crohn's disease, compared to

an end-to-end anastomosis. This was shown in a meta-analysis (He *et al.*, 2014); 396 patients with a side-to-side had a lower risk of recurrence compared to 425 patients with an end-to-end anastomosis (OR 0.20; 95% CI 0.07-0.55). However, the risk of recurrence of CD activity after stoma formation vs surgical anastomosis has been less widely studied. In 1991, Rutgeert and colleagues (Rutgeerts *et al.*, 1991) analysed the risk of recurrence of CD activity in 5 patients who had previously had a bowel resection and ileocolonic anastomosis and a proximal ileostomy to divert the faecal stream. Ileocolonoscopy and biopsies were performed 6 months later and then the patients underwent re-anastomosis surgery and again 6 months later. There were 75 control patients who underwent a resection and ileocolonic anastomosis in one step. None of the 5 patients with an ileostomy had endoscopic recurrence in the neo-terminal ileum compared to 53 out of 75 patients in the control group. After re-anastomosis, all 5 patients had endoscopic recurrence. This implicates the anastomosis and the faecal stream's role in recurrence of inflammation (Rutgeerts *et al.*, 1991). Another study of 112 patients (Ozgur *et al.*, 2021) found that creation of a stoma was associated with a reduced risk of recurrence, however, when multivariate analysis was performed this was no longer significant. Our study supports findings that an enterostomy may confer a lower risk of recurrence of inflammation. This risk factor should be considered when making decisions regarding the type of surgery to perform, or whether re-continuity surgery should take place, in cases where a patient has a borderline bowel length.

It would be important to consider differences in post operative medication use in the enterostomy group compared to the CIC group as this is a confounder. I am planning to carry this out in future work.

3.5.2 Upper gastrointestinal disease

Patients with UGI disease represent a more severe cohort and therefore are more likely to have ongoing active disease. In the active disease group, 17.6% had UGI disease compared to 4.8% in the remission 1 year group (P=0.01) (Table 8). UGI was also shown to be a risk factor for active disease in the 3 years (Table 9) and 5 years (Table 10) remission groups.

3.5.3 Penetrating disease

Penetrating disease at follow up was more common in patients with active disease compared to those in remission for the previous 5 years (Table 10). Penetrating disease is an established risk

factor for post-operative recurrence of CD (Chen *et al.*, 2019; Riss *et al.*, 2013; Khoshkish *et al.*, 2012; Rutgeerts *et al.*, 1990a; Lee *et al.*, 2012)

3.6 Conclusion

The rate of 1-year remission of the CD-IF group was double that of the CD group (75.5% vs 37%). Only 8.6% of CD-IF group were currently requiring biologic medications at their latest clinic compared to 48% in the CD group. This shows that Crohn's disease activity appears to burn out after IF develops; possible explanations include a change in the microbiota related to dietary changes or parenteral nutrition, insufficient bowel length to inflame or due to anatomical changes which may allow oxygen tolerant or bile tolerant bacteria to thrive. Also, the lack of a surgical anastomosis through the formation of end enterostomy, which is a risk factor for inflammation, may also contribute to remission.

The risk factors for active disease in the CD-IF cohort was the presence of an anastomosis, presence of UGI disease and penetrating disease. The presence of perianal disease and EIMs were not risk factors for CD-IF. Smoking and family history were not assessed due to missing data. Although the overall cohort of CD-IF did not have very active disease, patients with CD-IF should still be regularly monitored for active disease, especially if they have an anastomosis, UGI disease or penetrating disease.

Chapter 4 Literature reviews of multiomics in Crohn's disease and Intestinal Failure

4.1 Microbiota in Crohn's disease

Multiple meta-analyses have been published regarding the microbiota changes in IBD; therefore, literature review was not undertaken. There is a distinct Crohn's disease microbiota 'signature' which has been consistently demonstrated in the published literature. Changes also appear to be associated with more active disease. The most consistent changes include a decreased diversity (Shen *et al.*, 2017); decreases in the *Firmicutes* phylum (specifically Roseburia and F. prausnitzii); increases in the *Proteobacteria* phylum (eg. *E.Coli* and *Enterobacteriaceae*) (Swidsinski *et al.*, 2002; Sokol and Seksik, 2010) and decreases in the *Bacteroidetes* phylum (eg. *Bacteroides*); and increases in the *Actinobacter* phylum (eg. *Bifidobacteriaceae*)(Matsuoka and Kanai, 2015; Zhang, Wang and Miao, 2017). Overall, there is a decrease in 'good' or anti-inflammatory bacteria, for example, *Faecalibacterium* prausnitzii and bacteria that produce short chain fatty acids (SCFAs) and an increase in pro-inflammatory bacteria, specifically, adherent-invasive *E.Coli* is implicated as being critical in the pathogenesis of IBD.

There are large discrepancies in different findings due to the nature of the effect of confounders and study design. Studies continue to add to the wealth of data to build up reliable information and understanding about the disease.

It has been long debated whether alterations in the microbiome contribute to the pathogenesis or are simply a secondary effect of IBD. Studies in the last decade had provided evidence that a disturbed microbiome contributes to the pathogenesis of IBD, or is a trigger for, rather than being a consequence of IBD. In the widely quoted study, Sokol and colleagues (Sokol *et al.*, 2008) analysed the microbiota in ileal resection specimens and showed that patients with a lower *F.prausnitzii* abundance in the initial surgical resection specimen were at higher risk of post-operative recurrence 6 months later. Since then, a meta-analysis by Cao and colleagues (Cao, Shen and Ran, 2014) in 2014 in IBD included 11 studies with 1180 patients and showed that the abundance of *F.prausnitzii* was significantly lower in IBD compared to healthy controls and the effect was greater in CD compared to controls. Fujimoto and colleagues (Fujimoto *et al.*, 2013) compared the stool microbiota of 47 CD patients and 20 healthy controls; F. prausnitzii was lower in the CD group. Within the CD group, those with higher *F.prausnitzii* abundance had a lower Crohn's disease activity index, a lower c-

reactive protein (CRP) and erythrocyte sedimentation rate and a high albumin which is in keeping with previous studies. *F.Prausnitzii* is known to produce butyrate (Hold *et al.*, 2003).
Studies in paediatric patients (Lewis *et al.*, 2015) have demonstrated that the degree of dysbiosis after treatment reduces in responders but not in non-responders. Modulation of the microbiota can be an effective therapy in IBD. For example, studies (Schultz, 2008; Henker *et al.*, 2008; Kruis *et al.*, 2004) have shown that *Escherichia Coli* Nissle 1917 probiotic therapy has similar efficacy in maintaining remission compared to mesalazine therapy. A randomised controlled trial (RCT) of 29 paediatric patients (Kruis *et al.*, 2004) showed that a probiotic preparation (VSL3) was associated with a significantly lower relapse rate compared to placebo. Further support comes from 2 meta-analyses which demonstrated that certain antibiotics are efficacious in CD (Wang, Wang and Yang, 2012; Khan *et al.*, 2011). A RCT (Tamaki *et al.*, 2016)of 56 patients with UC treated with *Bifidobacterium* or placebo showed a decreased UC disease activity index in the probiotic group, but not in the placebo group. (Schultz, 2008). In a meta-analysis (Narula *et al.*, 2017) of 4 RCTs with 277 patients who received FMT treatment for active UC, patients treated with FMT had a higher remission rate compared to the placebo group. A study by Jewel and colleagues (Harper *et al.*, 1985) highlighted the role of the faecal stream on the maintenance of inflammation in 22 patients with Crohn's colitis who had a defunctioning loop ileostomy at least 6 months prior. Small bowel effluent or ultrafiltrate were transferred into the defunctioned colon. With transfer of the ultrafiltrate, there was little difference, but with transfer of the ileostomy effluent, there were marked differences in 8 out of 15 patients; with one patient relapsing with pain and vomiting, 2 had abdominal pain and 1 had abdominal pain and diarrhoea and 1 developed oral apthoid ulceration, 2 developed perianal irritation (one of whom developed an abscess). The effects in the effluent group occurred mostly within 1 week and persisted for the duration of the study. A prospective study (Ananthakrishnan *et al.*, 2017) assessed disease activity and stool microbiota in CD and UC patients initiating Vedolizumab at week 0, 14, 30 and 54. Changes were seen in week 14 responders including: an increase in alpha diversity and an increase in Roseburia inulinvorans and Burkholderiales. Changes seen at 14 weeks, persisted to 54 weeks in responders.

4.1.1 Role as a predictive tool in CD

There have been several studies highlighting the potential role of using microbiota data as biomarker tools in Crohn's disease assessment:

Study	Patient groups	Finding
Sokol (Sokol *et al.*, 2008)	Biopsy samples from initial resection specimen	Patients with a lower *F. prausnitzii* in the initial surgical resection specimen had a higher risk of

		post-operative recurrence. The same group then administered oral live *F. prausnitzii* to a mouse model of chemical induced colitis; there was a reduction in the severity of the colitis. In cellular models, metabolites produced by F. prausnitzii are able to block NF-kB activation and IL-8.
Mondot (Mondot *et al.*, 2016).	Mucosal microbiota from 20 ileal resection samples were assessed at baseline and again at 6 months.	Patients who had a recurrence had a higher abundance of *E.durans* at 6 months
Pascal (Pascal *et al.*, 2017)	2045 non-IBD and IBD faecal samples	A signature of 8 microbes to detect Crohn's disease with a sensitivity of 80% including *Faecalibacterium, Peptostreptoccoccaceae, Anaerrostipes, Methanobrevibacter, Christenellaceae, Collinsella* and *Fusobacterium and Escherichia*
(Kolho *et al.*, 2015)	32 paediatric patients	Those with greater inflammation had reduced microbial richness and reduced abundance of butyrate producers. In those who started anti-TNF therapy, the microbial diversity increased in the responder group by 6 weeks ($p<0.01$). 6 groups (eg. *Eubacterium rectale* and *Bifidobacterium*) predicted the response to medication
Racja (Rajca *et al.*, 2014)	Assessed faecal samples from 33 CD patients at 0, 2 and 6 months	*F.prausnitzii* and *Bacteroides* were less abundant in relapsers than in those who maintained remission.
Ding (Ding *et al.*, 2020b)	76 CD patients commencing anti-TNF therapy, there were 11 responders, 37 non-responders and 28 partial responders.	Histidine and cysteine were identified as biomarkers of response and primary bile acids were associated with non-response to anti-TNF therapy; with higher levels of phase 2 conjugated bile acids in non-responders.

4.2 Literature review of microbiota in intestinal failure

Unlike the large body of evidence that exists regarding the microbiota in CD, there have been fewer studies examining the microbiota in patients with intestinal failure. The surgical procedure in itself alters the microbiota as it leads to a change in the luminal oxygen exposure, blood flow changes, and changes in the pH towards more acidic pH, transit time is faster and biliary and pancreatic secretions can pass into the colon and it leads to greater gram-positive bacteria, for example, the facultative anaerobe, *Lactobacillus*. Changes seen after bowel resection in humans can be a consequence of the surgery, the underlying disease or due to changes in diet and parenteral nutrition; these confounders can cause inconsistent results.

4.2.1 Search strategy for identification of studies

A literature review examining the changes in the microbiota in IF was conducted according to the PRISMA guidelines (Moher *et al.*, 2009), using the search terms

Books@Ovid <June 12, 2023>

Journals@Ovid Full Text <June 23, 2023>

Imperial Journals@Ovid

Embase Classic+Embase <1947 to 2023 June 23>

Global Health <1973 to 2023 Week 25>

HMIC Health Management Information Consortium <1979 to March 2023>

Ovid MEDLINE(R) ALL <1946 to June 22, 2023>

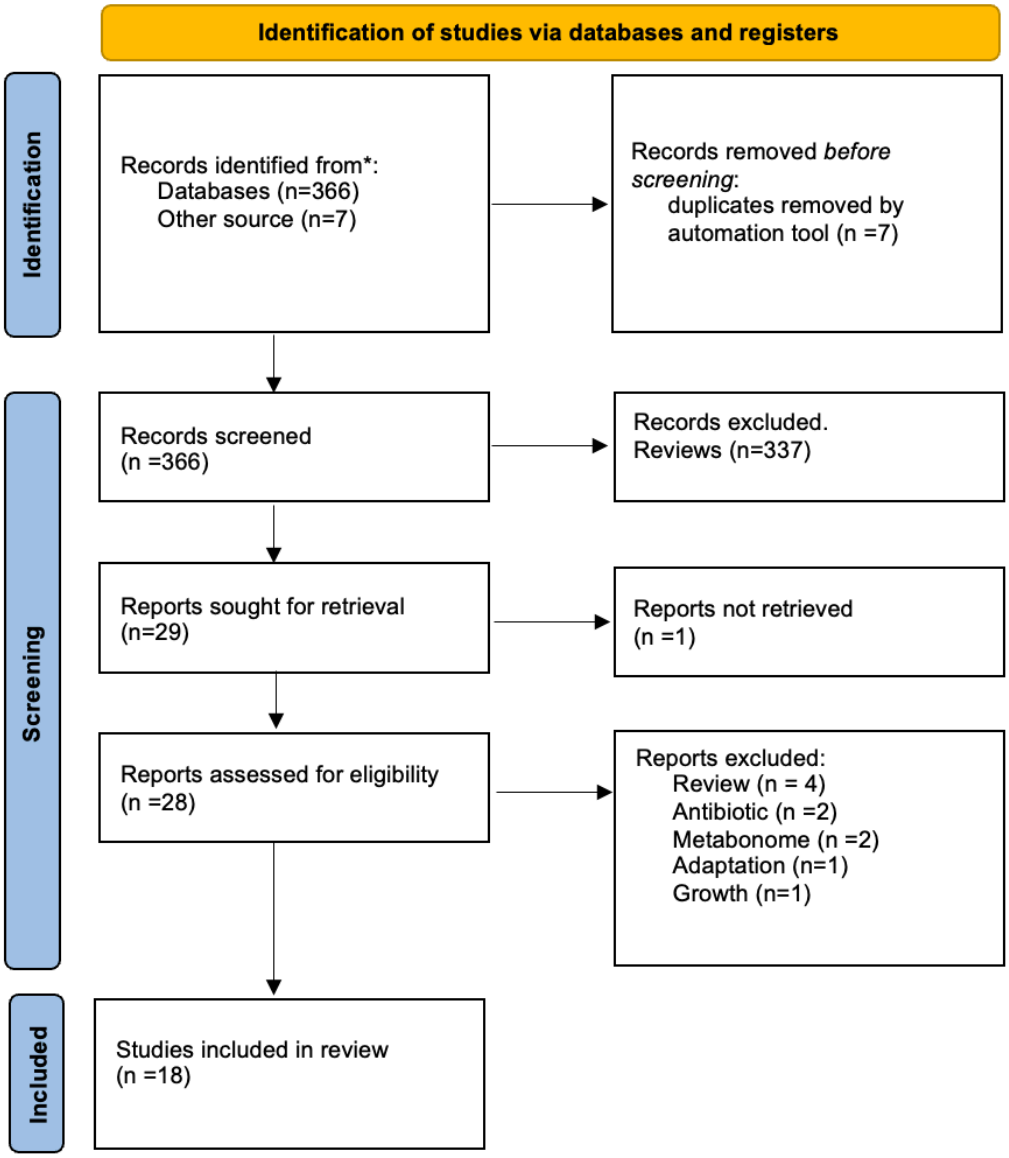
APA PsycInfo <1806 to June Week 3 2023>

Transport Database <Pre-1987 to June 2023>

1	(microbiom* or microbiota or 16s).ab.	463516
2	((short bowel or intestinal failure) not transplan*).ab.	13103
3	1 and 2	370

370 results were returned, of which 37 full texts were screened. 16 studies were included Table 11 shows the findings in all IF studies. (Figure 1).

Figure 1. PRISMA diagram showing selection of studies for studies on the CD metabonomic profile



4.2.2 Summary of microbiota signatures in intestinal failure

18 published studies assessing the microbiota in SBS were identified: 3 were in animals, 8 studies were in children and 7 were in adults (Table 11).

Amongst the adult human studies, most patients lacked an ileocaecal valve (ICV). The human studies all consisted of patients of mixed SBS aetiology, and the largest study consisted of 16 SBS patients and the largest number of CD patients was 4 (Boccia *et al.*, 2017).

In the paediatric studies there was a **decrease in diversity** (Zeichner *et al.*, 2019) (Engstrand *et al.*, 2015), **increase in Lactobacilli** (Korpela *et al.*, 2017) (Davidovics *et al.*, 2016), **reduced anaerobes** (Piper *et al.*, 2017b) and **increased *Proteobacteria*** (Piper *et al.*, 2017b) (Ralls, Miyasaka and Teitelbaum, 2014) (Engstrand *et al.*, 2015) (Davidovics *et al.*, 2016).

The most consistent finding amongst the adult studies with patients with SBS of mixed causes mirrored the findings in the paediatric studies and include **reduced overall diversity**, **increased *Lactobacilli***, **increased *Proteobacteria***, such as *Escherichia Coli* and *Enterobacteriaceae*, **decreased anaerobes** such as *Clostridium leptum*, and **reduced *Bacteroidetes***. In a study (Budinska *et al.*, 2020), patients with SBS had a reduced diversity and reduction of *Bacteroidetes* and an increased in *Lactobacillaceae* and *Enterobacteriaceae* compared to healthy controls. Joly and colleagues (Joly *et al.*, 2010) examined the microbiota of both faecal samples and mucosa; both had an increase in *Lactobacillus* which is a consistent finding amongst animal, paediatric and adult studies.

Animal studies in mice(Sommovilla *et al.*, 2015b; Devine *et al.*, 2013) and piglets show an increased in *Lactobacillus* and a reduced diversity (Sommovilla *et al.*, 2015b) which is in keeping with human studies. *Bacteriodetes* were decreased (Devine *et al.*, 2013).

There are several possible explanations for these consistent changes seen after bowel resection, as discussed in section 6.9, including: increased transit time, increased oxygen in the luminal environment, increased pH and increase in undigested content which occurs after intestinal resection resulting in a short bowel.

Table 11 Animal and human studies showing the microbiota changes in short bowel.

ICV, ileocaecal valve; NEC necrotising enterocolitis

Author	**Patients/cause of short bowel**	**Anatomy**	**Parenteral nutrition (%)**	**Findings**
Animal studies				
Sommovilla (Sommovilla *et al.*, 2015a)	14 SBS mice 50% small bowel resection at 8 weeks			No difference in diversity. SBS mice – **increased *Lactobacillus*** and **decreased *Enterobacteriaceae*** from the ileum

	12 controls (sham operation)			
Devine (Devine *et al.*, 2013)	Mice ileocaecal resection Non ICR controls			**Increased *Firmicutes*, decreased *Bacteroidetes***
Lapthorne (Lapthorne et al., 2013)	12 SBS piglets 75% bowel resection at 4 weeks 10 sham operation, 12 No surgery			**Decreased *Firmicutes*, decreased diversity**
Paediatric studies				
Ralls (Ralls, Miyasaka and Teitelbaum, 2014)	12 undergoing bowel resections			In patients with enteral deprivation, there was increased *Enterobacter, Shigella, Klebsiella* and *Fusobacterium*
Korpela (Korpela *et al.*, 2017)	23 patients 58 healthy controls			Patients with IF had increased Lactobacilli, *Proteobacteria* and *Actinobacteria*. *Clostridium clusters III, IV, and XIVa* were reduced
Engstrand (Engstrand *et al.*, 2015)	11 SBS children (6 NEC, 2 atresia, 2 volvulus, 1 gastroschisis) 7 controls healthy siblings	4/11 had ICV.	6 off PN 5 on PN	**Reduced diversity and increased *Enterobacteriaceae*** of those on PN compared to those weaned off PN and healthy controls.
Davidovics (Davidovics *et al.*, 2016)	9 SBS children (3 NEC, 4 gastroschisis, 1 volvulus, 3 atresia), 8 controls	3/9 had ICV.	Calories from PN was 25% (0-100%)	*Firmicutes* were the most abundant. **Increased *Proteobacteria* (e.g., *Escherichia Coli*)** made up 22% of the SBS samples compared to only <1% of the control samples. Those with increased stool frequency had an abundance of ***Lactobacillus***

Piper (Piper *et al.*, 2017a)	8 SBS children (5 NEC, 2 SB atresia, 1 gastroschisis, 1 tumour 3 controls	3/8 had ICV.	3 off PN 5 on PN	Marked dysbiosis with **reduction in anaerobes.** **Increased *Enterobacteriaceae***
Zeichner (Zeichner *et al.*, 2019)	25 SBS children (7 NEC, 5 gastroschisis, 8 atresia, 4 volvulus, 1 Hirschsprung's) 4 controls	8/25 had ICV.	16 off PN 9 on PN and enteral feeding	Lower diversity compared to controls especially those with enteral deprivation and without an ICV. **Higher** stool ***Bifidobacterium, Staphylococcus, Veillonella*.**
Thanert 2021 (Thänert *et al.*, 2021)	19 SBS children and 19 controls			Increased ***Enterococcus*** and decreased ***Ruminococcus***, *Bifidobacterium*, *Eubacterium,* and *Clostridium* species.
Neelis 2022 (Neelis *et al.*, 2022)	15 IF 3 atresia 2 gastroschisis 2 NEC 1 hernia strangulation 2 pseudo-obstruction 1 microvillus inclusion disease 1 Protein losing enteropathy 1 tricho-hepato- enteric syndrome 1 esophageal atresia with motility problems 1 unknown cause. 25 healthy controls	9/15 had ICV	15 on PN	**Reduced diversity** **Increased *Escherichia-Shigella*** **Increased *Staphylococcus*** **Decreased *Faecalibacterium*** **Decreased *Ruminococcus***
Adult human studies				
Joly(Zeichner *et al.*, 2019)	11 SBS adults (8 mesenteric infarct, 1 volvulus **1 Crohn's**)	0 had ICV.	None on PN	**Increased *Lactobacillus***

	8 controls		Large oral intake	**Decreased *Clostridium leptum*, *Clostridium coccoides* and *Bacteroidetes*.**
Mayeur (Mayeur *et al.*, 2013)	16 SBS type 2 adults (11 mesenteric infarct, 2 radiation enteritis, 1 trauma, 1 caustic, 1 surgical) No controls	0 had ICV	Oral intake only	**Increased *Lactobacillus*** **Decreased *Clostridium* and *Bacteroides***
Furtado (Furtado *et al.*, 2013)	7 SBS patients (7 mesenteric ischaemia) 7 controls	4 had ICV	All on PN	**Higher *Enterobacteriaceae* (*Proteobacteria*)**
Boccia (Boccia *et al.*, 2017)	12 SBS patients (5 mesenteric ischaemia, **2 Crohn's**, 2 adhesions, 1 congenital) 16 controls	2 had ICV	all on PN	**Decreased *Bacteroidetes* and *Firmicutes*** **Increased *Lactobacillus***
Huang (Huang *et al.*, 2017)	5 SBS type II patients (2 surgical, 2 volvulus, 1 internal hernia) 5 SBS type III patients (2 surgical, 1 volvulus, 1 mesenteric ischaemia) 5 controls	5 had ICV	8 on PN All enteral	**Reduced diversity** correlated with small bowel length. SBS II – **Increased *Enterobacteriaceae* (*Proteobacteria*), decreased *Firmicutes***. In SBS III, **increased *Lactobacillus* and *Bacteroidetes*** **Duration of PN correlated with abundance of *Enterobacteriaceae* but negatively with *Lactobacillus*.**
Boutte (Boutte *et al.*, 2022)	22 patients with SBS 8 ischaemic bowel, 1 congenital, 2 malignancy, 2 obstruction, 3 enterocutaneous fistula,		22 on PN	**Reduced diversity.** **Increased *Lactobacillus*** **Increased *Enterobacteriaceae***

	2 radiation enteritis, **4 Crohn's disease**			
Budinska 2020 (Budinska *et al.*, 2020)	24 with SBS 5 with jejunocolic anastomosis but no PN 40 healthy controls	0 /24 had ICV	24 on PN	**Reduced *Bacteroidetes*** **Increased *Proteobacteria*** **Increased *Lactobacteriaceae*** **Increased *Enterobacteriaceae*** **Reduced *Ruminococcaceae***

4.3 Literature review of metabonomic profile in Crohn's disease

Metabonomics is the quantitative study of the metabolic profile measured in biofluids including faeces, serum, urine of complex systems, for example, mammals (Marchesi and Ravel, 2015) and Is studied using NMR or MS. Metabonomic studies have become increasingly performed since 2007 in CD. However, there is a lack of data on the metabonomic profile in patients with CD-IF. Small bowel resection affects the luminal environment; there is an increased transit time, a higher oxygen environment and reduced surface area for reabsorption of nutrients and bile acids; these changes impact the metabonome as well as the microbiome. Metabonomics has a role in the assessment and monitoring of IBD as it is non-invasive, it can also provide insight into disease pathogenesis. It can cross link metabolites to specific bacteria and advance the understanding on the interplay between the host and the microbiota. The gut microbiota can produce or affect the metabolism of metabolites which can influence intestinal homeostasis. Reciprocally, changes in the anatomy and surface area can lead to changes in metabolites which can impact the microbiota.

The inconsistencies and number of different changes reported highlight the complexity of metabonomic studies and the high throughput nature of the techniques which are sensitive to patient factors and sample factors (section 1.2). Some of the metabolite changes are not reproduced in different studies, however we performed a literature review and select the consistent findings.

4.3.1 Search strategy for identification of studies

A literature review to determine the metabonomic changes in CD was performed using the search terms below which returned 371 articles (Figure 2).

termsBooks@Ovid <June 26, 2023>

Journals@Ovid Full Text <June 30, 2023>

Imperial Journals@Ovid

Embase Classic+Embase <1947 to 2023 June 30>

Global Health <1973 to 2023 Week 26>

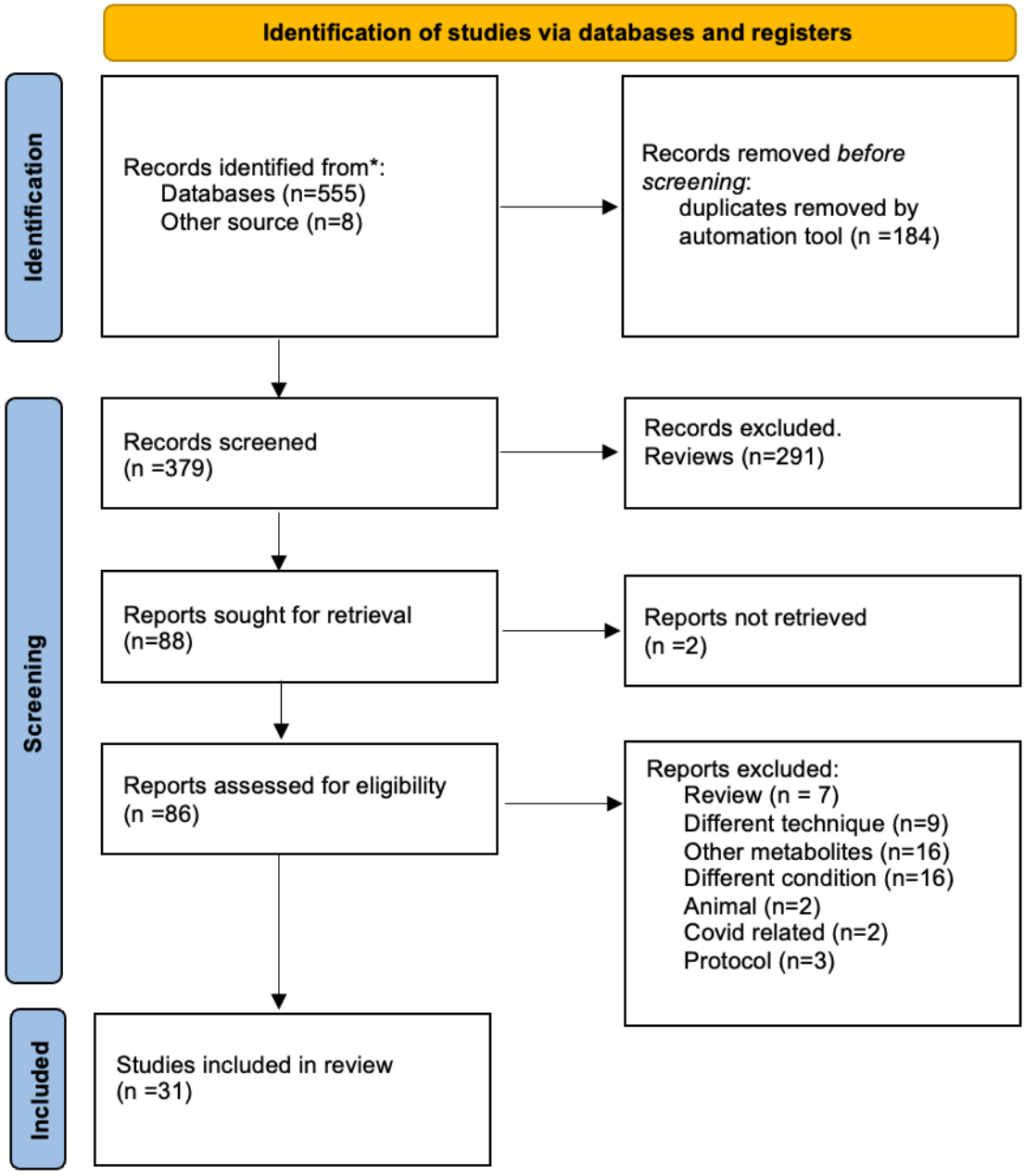
HMIC Health Management Information Consortium <1979 to May 2023>

Ovid MEDLINE(R) ALL <1946 to June 29, 2023>

Transport Database <Pre-1987 to June 2023>

1	((Crohn* or ibd or inflammatory bowel disease) not mice not murine).ab.	260041
2	(stool or faeces or feces or faecal or fecal or urin* or serum or plasma).af.	11138321
7	metabo*om*.ab.	129750
8	1 and 2 and 7	638
10	limit 9 to humans	555
11	remove duplicates from 10	371

Figure 2 PRISMA diagram showing selection of studies for studies on the CD metabonomic profile



4.3.2 Summary of metabonomic profile in Crohn's disease

31 studies were included in the final analysis; bile acid and fatty acid changes AR were included in Table 12. There were 16 altered key bile acids and 7 altered key short chain fatty acids that are emerging as the key players in the IBD metabonome.

Table 12 Changes in key metabolites in Crohn's disease compared to healthy controls in urine, stool and serum a) bile acids, b) short chain fatty acids

	Urine	Stool	Serum
A) Altered bile acids			
Upregulated Bile acids		**Primary bile acids:** **Cholic acid** (Franzosa *et al.*, 2019; Lloyd-Price *et al.*, 2019) (Budinska *et al.*, 2020) (Jansson *et al.*, 2009) (Das *et al.*, 2019) **Glycocholic acid** (Kolho *et al.*, 2017), (Das *et al.*, 2019) **Taurocholic acid** (Bushman *et al.*, 2020) (Das *et al.*, 2019) **Chenodeoxycholic acid** (Franzosa *et al.*, 2019; Jansson *et al.*, 2009) (Das *et al.*, 2019) **Taurochenodeoxycholic acid** (Chen *et al.*, 2022)	**Primary bile acids:** **Cholic acid** (Gnewuch *et al.*, 2009) (Budinska *et al.*, 2020; Scoville *et al.*, 2018a) **Taurocholic acid** (Scoville *et al.*, 2018a), (Budinska *et al.*, 2020) **Chenodeoxycholic acid** (Gnewuch *et al.*, 2009),(Budinska *et al.*, 2020) **Glycocholic acid** (Scoville *et al.*, 2017; Wilson *et al.*, 2020) , (Budinska *et al.*, 2020) **Taurochenodeoxycholic acid** (Chen *et al.*, 2022), **Glycochenodeoxycholic acid** (Scoville *et al.*, 2018a),
Downregulated Bile acids	**Secondary bile acids:** **Glycohyodeoxycholic acid** **Glycolithocholic acid**	**Secondary bile acids:** **Lithocholic acid** (Franzosa *et al.*, 2019; Feng *et al.*, 2022; Wang *et al.*, 2021) (Budinska *et al.*, 2020; Bushman *et al.*, 2020) (Das *et al.*, 2019) (Budinska *et al.*, 2020)	**Secondary bile acids:** **Glycolithocholic acid sulphate** (Scoville *et al.*, 2018a) **Lithocholic acid** (Wilson *et al.*, 2020; Budinska *et al.*, 2020) **Deoxycholic acid**(Roda *et al.*, 2019) (Budinska *et al.*, 2020)

		Deoxycholic acid 20 (Franzosa *et al.*, 2019; Wang *et al.*, 2021; Bushman *et al.*, 2020) (Weng *et al.*, 2019) (Das *et al.*, 2019) **Hyodeoxycholic acid** (Wang *et al.*, 2021)	
B) Altered short chain fatty acids			
	Urine	**Stool**	**Serum**
Upregulated SCFAs		**Lactate** (Williams *et al.*, 2012b; Martin *et al.*, 2017; Franzosa *et al.*, 2019)	
Downregulated SCFAs	**Acetate** (Kolho *et al.*, 2017) 2 **hydroxybutyrate** (Martin *et al.*, 2017)	**Acetate** (Franzosa *et al.*, 2019; Powles *et al.*, 2019; Klassen *et al.*, 2017; Marchesi *et al.*, 2007; Treem *et al.*, 1994) (Marchesi *et al.*, 2007; Hove and Mortensen, 1995; Tjellström *et al.*, 2012; Takaishi *et al.*, 2008) **Butyrate** (Bjerrum *et al.*, 2015a; Aden *et al.*, 2019; Franzosa *et al.*, 2019; Lloyd-Price *et al.*, 2019) (Marchesi *et al.*, 2007; Hove and Mortensen, 1995; Tjellström *et al.*, 2012; Takaishi *et al.*, 2008)	**Acetate** (Yau *et al.*, 2013) **Butyrate** (Powles *et al.*, 2019) **hydroxybutyrate** (Scoville *et al.*, 2018a) (Schicho *et al.*, 2012)

		3-hydroxybutyric acid (Santoru *et al.*, 2017) **Propionate** (Bjerrum *et al.*, 2015a) **Valerate** (Marchesi *et al.*, 2007; Hove and Mortensen, 1995; Tjellström *et al.*, 2012; Takaishi *et al.*, 2008)	

4.3.3 Bile acid physiology

During normal bile acid enterohepatic recycling, the predominant primary bile acids (PBAs): cholic acid (CA) and chenodeoxycholic acid (CDCA), undergo conjugation with glycine or taurine by liver hepatocytes (148) and this step increases their water solubility for their transport in bile and they form excretory micelles which are complexes with cholesterol and other lipophilic lipid to facilitates their excretion via bile into the small bowel. 95% of conjugated PBAs are reabsorbed actively into the terminal ileum via the apical-sodium bile acid transporter (ASBT)(Thomas *et al.*, 2022).

The 5% unabsorbed PBAs enter the colon undergo several changes, of which the main two steps are deconjugated by bile salt hydrolase (BSH) enzymes possessed by the colonic microbiota, and 7a dehydroxylation to form secondary bile acids (SBAs). The main SBAs are deoxycholic acid (DCA) and lithocholic acid (LCA). Ursodeoxycholic acid is a less abundant SBA which is produced when CDCA undergoes deconjugation and epimerisation of the 7 α-hydroxyl group. The modification made to bile acids leads to several bile acid derivatives which have roles in microbiota- host signalling.

SBAs, which make up 80-90% of faecal bile acids, are passively reabsorbed in the colon and transported to the liver where they are conjugated with glycine and taurine and secreted into the lumen (149).

BAs bind to and activate the farnesoid X receptor (FXR); CDCA has the greatest affinity for FXR. The FXR is present in multiple organs, but it is most abundant in the terminal ileum. Upon bile acids binding to FXR, FXR reduces absorption of PBAs by downregulating the apical sodium- dependent bile acid transporters. FXR activation also stimulates the release of fibroblast growth factor 19

(FGF19) which has a negative feedback control on hepatic BA synthesis. FXR has a role in modulating inflammation. FXR agonists have been shown to attenuate the severity of chemical induced colitis in mice and reduce TNF alpha expression; this improvement was not seen in FXR knockout mice (Vavassori *et al.*, 2009).

4.3.3.1 Bile acids changes in CD compared *to controls.*

The most consistent findings are (Table 12):

- Stool primary bile acids (PBAs) such as cholic acid and chenodeoxycholic acid are increased, as are their conjugated derivatives glycocholic acid and taurocholic acid.
- Serum PBAs are upregulated, which is unexpected given that there is a reduced reabsorption. One proposed theory is that as a result of reduced reabsorption, there is a compensatory increase in hepatic synthesis of primary bile acids via s reduction in FGF19.
- Stool SBAs such as lithocholic acid and deoxycholic acid are lower in those with active CD compared to healthy controls or those in remission. One study showed a positive correlation between the length of ileum resected and the faecal PBA levels. Furthermore, bacteria responsible for converting PBAs into SBAs are present mainly in the colon.

The underlying mechanisms responsible for the increased serum PBAs changes are discussed in **section 6.9** and include reduced ileal length resulting in reduced FXR, therefore reduced FGF-19 and a loss of the negative feedback resulting in increased hepatic synthesis.

SBAs are thought to be anti-inflammatory through binding to the FXR receptor which leads to the inhibition of proinflammatory cytokines tumour necrosis factor-α (TNF-α), nuclear factor-kappa B (NF-κB), IL-1 and IL-6.

There is also altered microbiota therefore reduced transformation of PBAs to SBAs. PBAs exert pro-inflammatory effects on the intestines and SBAs exert anti-inflammatory effects.

In ileal CD, there is thought to be loss of farnesoid X receptor (FXR) due to loss of ileum. s. In support of this idea are studies which show that there is a decrease in FGF-19 levels in patients with short bowel syndrome (SBS) (Mutanen *et al.*, 2015). In another study (Boutte *et al.*, 2022) SBS patients had significantly lower levels of FGF-19 levels compared to healthy controls, in keeping with

other studies. There was no difference in FGF-19 between those with colon in continuity and those with a small bowel enterostomy. The stool CA and CDCA were elevated in patients with SBS whose colons were in continuity compared to those with an enterostomy. Stool conjugated PBAs were lower in patients with SBS with a colon as colonic bacteria can deconjugate BAs (Boutte *et al.*, 2022). An alternative theory, as suggested by one author (Boutte *et al.*, 2022) has suggested that the reason for the raised serum PBAs in SBS may be due to colonic absorption of BAs because serum PBAs were higher in SBS with a colon compared to without a colon, but this did not reach significance.

4.3.3.2 Bile acid changes comparing active disease vs remission.

The studies examined the metabonome in CD compare active disease to inactive CD. The most consistent changes distinguishing active CD and remission CD are:

1. Faecal SBA level were higher in heathy controls and patients with CD treated with anti-TNF therapy than in patients with CD treated with conventional therapy. Anti-TNF therapy restored SBA levels in the serum, especially deoxycholic acid which is absorbed passively (Roda *et al.*, 2019).
2. Another study of 76 patients with CD showed that PBAs were associated with non-response to anti-TNF therapy and there was an increase in sulphate and glycine conjugated primary bile acids in patients who did not respond to anti-TNF therapy (Ding *et al.*, 2020a).

SBAs, which make up 80-90% of faecal bile acids, are passively reabsorbed in the colon and transported to the liver where they are conjugated with glycine and taurine and secreted into the lumen (Tiratterra *et al.*, 2018).

SBAs are thought to be anti-inflammatory through binding to the FXR receptor which leads to the inhibition of proinflammatory cytokines tumour necrosis factor-α (TNF-α), nuclear factor-kappa B (NF-κB), IL-1 and IL-6(Ding *et al.*, 2015).

There is also altered microbiota therefore reduced transformation of PBAs to SBAs (5). PBAs exert pro-inflammatory effects on the intestines and SBAs exert anti-inflammatory effects.

4.3.4 Short chain fatty acid changes

Short chain fatty acids (SCFAs) are produced by bacterial fermentation of fibres from the diet and undigested complex carbohydrates. A reduction in SCFAs in CD is thought to be due to a reduction in SCFA producing bacteria. SCFA producing bacteria include *Faecalibacterium, Clostridium* clusters IV and XIVb, *Roseburia*, and *Ruminococcus* (Svolos, Gkikas and Gerasimidis, 2021). SCFAs have several desirable functions: they are an energy source for colonic cells. Butyrate and acetate have been shown to inhibit histone deacetylases (HDACs) and therefore decrease NF-kB induced proinflammatory cytokines such as TNFa, IL-6, IL12, iNOS and upregulate anti-inflammatory T regulatory cytokines such as IL-10. Evidence for this comes from a study by Segain and colleagues (Segain *et al.*, 2000) in which administration of butyrate enemas to CD patients led to decreased TNF in intestinal biopsies and it reduced lipopolysaccharide (LPS) induced expression of cytokines by peripheral blood mononuclear cells and also decreased LPS induced NF-kB transcriptional activity. Butyrate also decreased the severity of trinitrobenzene suphonic acid induced colitis in rats. In a study of 32 paediatric patients (Kolho *et al.*, 2015) receiving anti-TNF therapy, those with greater inflammation had reduced microbial richness and reduced butyrate.

In CD, there is a decrease in SCFA- producing bacteria (eg F. prausnitzii, Clostridium clusters) (Parada Venegas *et al.*, 2019) and there is an increase in fatty acid catabolism. The consistent findings in CD are that all the SCFAs are reduced in CD in urine, stool and serum. Marchesi and colleagues showed reduced stool SCFA levels in patients with IBD compared to control (Marchesi *et al.*, 2007) . There was also a decrease in acetate and butyrate in this study in CD compared to controls. A meta-analysis of 12 studies concluded that butyrate, acetate and valerate were reduced in CD but propionate was no different. Subgroup analysis according to disease state or location was not possible due to incomplete data. The SCFA which is consistently increased in CD in stool is lactate. Consistent findings include (Table 12):

- Reduced butyrate, especially in active disease (Bjerrum *et al.*, 2015b; Aden *et al.*, 2019; Franzosa *et al.*, 2019; Lloyd-Price *et al.*, 2019) (Marchesi *et al.*, 2007)
- Reduced stool acetate (Franzosa *et al.*, 2019; Powles *et al.*, 2019; A *et al.*, 2017) and reduced numbers of acetate producing bacteria.
- Reduced pyruvate (Klassen *et al.*, 2017)
- Reduced 2 hydroxybutyrate (Martin *et al.*, 2017)
- Reduced valerate.

- Increased lactate (Williams *et al.*, 2012b; Martin *et al.*, 2017). However in one study (Hove and Mortensen, 1995), compared to quiescent CD, lactate was increased in Crohn's colitis but not in isolated Crohn's ileitis.

4.4 Literature review of the metabonomic profile in intestinal failure

4.4.1 Search strategy for identification of studies

A literature review to determine the metabonomic changes in IF was performed using the search terms below which returned 89 articles, of which 3 were included in the final analysis (Figure 3).

Books@Ovid <July 10, 2023>

Journals@Ovid Full Text <July 13, 2023>

Imperial Journals@Ovid

APA PsycArticles Full Text

Embase Classic+Embase <1947 to 2023 July 13>

Global Health <1973 to 2023 Week 26>

HMIC Health Management Information Consortium <1979 to May 2023>

Ovid MEDLINE(R) ALL <1946 to July 13, 2023>

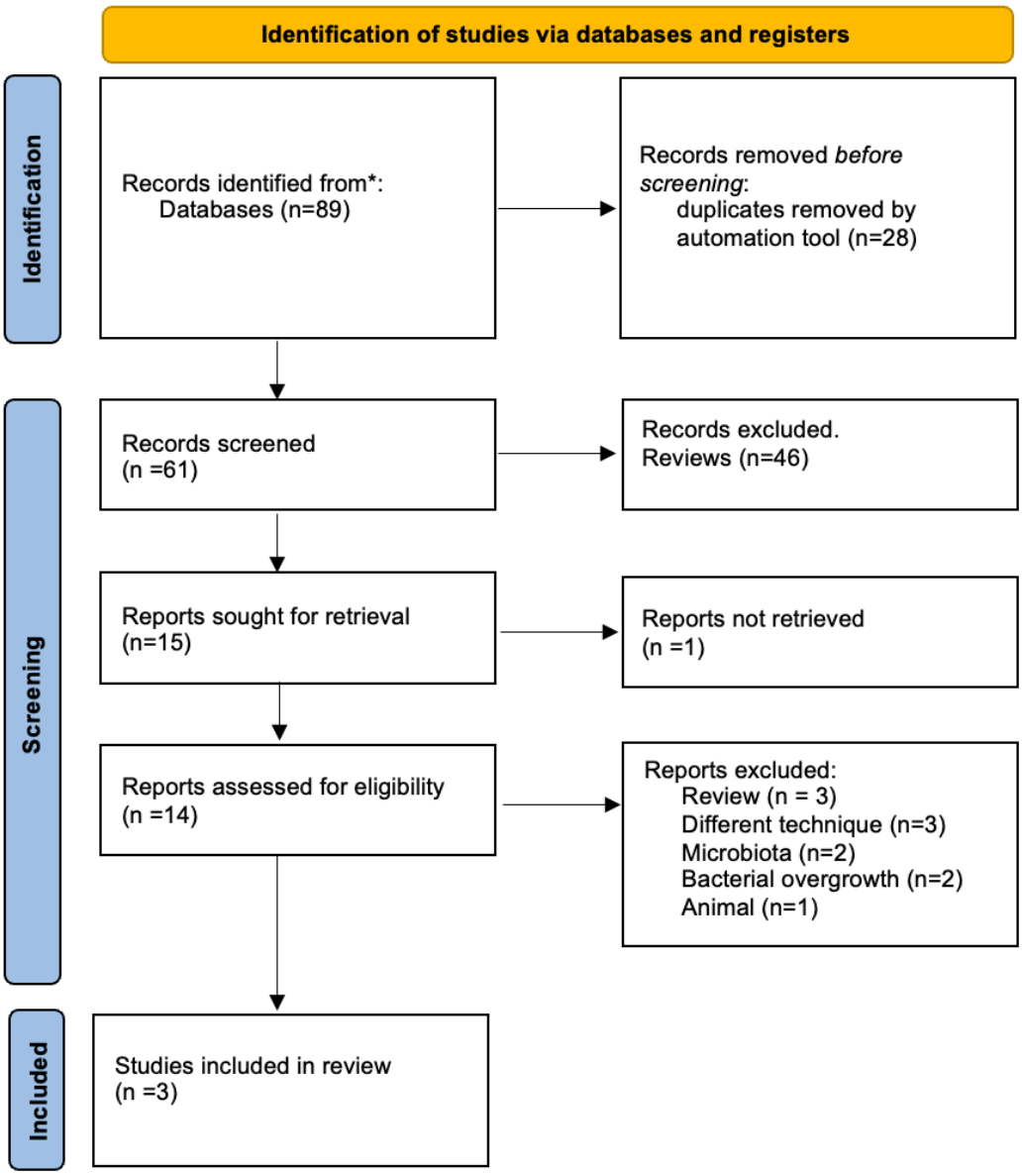
Maternity & Infant Care Database (MIDIRS) <1971 to July 04, 2023>

APA PsycInfo <1806 to July Week 2 2023>

Transport Database <Pre-1987 to July 2023>

1	(SHORT BOWEL or intestinal failure).ab.	17147
2	(metabo*om* or NMR or mass spectrometry).af.	2220048
3	(stool or faeces or faecal or feces or fecal or plasma or serum or urine).af.	10406183
4	(bile acid* or fatty acid* or cholic* or butyr*).af.	1476272
5	1 and 2 and 3 and 4	89

Figure 3 PRISMA diagram showing selection of studies examining the metabonome in Intestinal Failure



4.4.2 Summary of metabonomic profile in Intestinal Failure

Only a few studies have examined the metabonomic profile in IF. A study by Boutte (Boutte *et al.*, 2022) examined the metabonomic profile of 52 patients with short bowel syndrome (SBS), of whom 22/52 were dependent on parenteral nutrition (PN). There was an increase in the serum PBA:SBA ratio in the SBS group compared to healthy controls. Serum PBAs were higher in SBS patients with colon compared to SBS without a colon. There was a trend for increased serum CA and CDCA in SBS, largely attributable to SBS with colon. The authors suggested that there may be colonic absorption of BAs in SBS. There was greater serum glycocholic acid and taurocholic acid in SBS without colon,

which the authors proposed could be due to passive absorption through the bowel. In another study, (Budinska *et al.*, 2020) patients with SBS had higher faecal chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA) and a lower lithocholic acid (LCA) compared to controls; in the serum, cholic acid, glycocholic acid, CDCA were higher and DCA was lower (below the detection rate) compared to controls.

Chapter 5 Methods for microbiota and metabonomic studies

5.1 Ethics

This study is approved by REC 18/LO/1207 and has received local research and development approval.

5.2 Human studies inclusion criteria

A prospective cohort study. Participants attending Intestinal Failure clinics at St Mark's hospital between July 2018 - March 2019 were recruited and were provided a written patient information sheet (Supplementary material 1). Patients completed a metabonomics questionnaire (Supplementary material 2) which included dietary and lifestyle questions.

Inclusion criteria: $\geq$ 18 years old, IF due to small bowel CD, receiving home parenteral nutrition (HPN) or home intravenous fluids (HIVF) for more than one year.

Exclusion criteria: antibiotics or probiotics in the last 8 weeks, pregnant, active cancer, surgery in the last 6 months, colonic CD. Patients were defined as smokers if they had smoked more than 7 cigarettes per week in the last 1 year.

5.3 Sample collection

Samples were collected from patients on the day of the clinic. Samples were kept on ice for a maximum of 1 hour prior to processing for storage. Standardised operating procedures for collection and storage of samples were followed, to limit the factors which can affect the metabolic profile, for example, the time between sample production and storage, time in storage, and temperature of sample collection and storage.

5.3.1 Stoma effluent (stool) collection

1. Give a sterile container to the patient.
2. Ensure samples are collected on the same day.
3. Once the sample is produced in the container, insert sample into a cool bag which also contains an ice cooling pack.
4. Stool samples should be processed within 4 hours of being produced. Time the sample was produced should be noted at time of collection.
5. On collection, container to be labelled with patient's demographic details and the sample should be kept at a minimum of 4^{o}C until it is processed.

5.3.2 Serum collection

1. Label a plain blood collection bottle ('red top') was with patient's demographic details; collect approximately 5ml of whole blood was (usually at the same time as their clinical blood test).
2. Avoid vigorous shaking and unnecessary sunlight exposure.
3. The sample should be kept at a minimum of 4 ^{o}C (for a maximum of 4 hours) before processing.
4. Note the time the blood was drawn and processed.

5.3.3 Urine collection

1. Label a sterile Universal Container with patient's demographic details.
2. Avoid taking early morning urine; use mid-stream urine.
3. Keep the sample at a minimum of 4 ^{o}C (for a maximum of 4 hours) before processing.
4. Avoid unnecessary prolonged exposure to light.

5.4 Sample processing for storage

5.4.1 Stoma effluent (stool)

1. Work under a level 2 microbiology hood.
2. Clean the surface with 70% alcohol and distel.
3. Open and homogenise the stool in the container with a sterile spatula.
4.Label a minimum of 3 Eppendorf tubes for NMR, 3 for MS and 3 for 16s microbial processing with pseudonymised code.
5. Aliquot at least 1g of solid stool for MS, and 300mg for NMR and 250-300mg for 16s rRNA microbial profiling into each tube. Avoid food particles.
6. Freeze at -80^{o}C for a maximum of 24 months.

5.4.2 Serum

1. If the samples have been kept on ice, leave samples out for 30 mins to allow blood to clot; clotting time should not exceed one hour.
2. Make note of the time samples were processed as well.
3. Clean the surface with 70% alcohol and Distel.
4. Centrifuge the plain bottles at 1600g for 15 minutes at 4^{o}C.
5. Label Eppendorf tubes with patient's pseudonymised code.
6. Observe the bottles after centrifugation. If the supernatant is red/rose tinted, it is likely that the cells have haemolysed, and entire sample would have to be discarded.

7. If the supernatant is not red/rose colour, aliquot 400-600µl into a minimum of 3 labelled Eppendorf tubes.

8. Freeze at -80°C for a maximum of 24 months.

5.4.3 Urine

1. Assign a pseudonymised code for each patient.

2. Process one sample at a time.

3. Clean the surface with 70% ethanol and Distel.

4. Label Eppendorf tubes with pseudonymised code.

5. Transfer 1ml of urine into Eppendorf tubes and centrifuge at 13000g for 10 minutes at 4^0C.

6. Using a syringe filter, aliquot 600µl at a time of supernatant into further Eppendorf tubes (minimum 3) until no more supernatant left. Avoid disrupting the sediment following centrifugation in the first batch of Eppendorf tubes.

7. No additives are necessary.

8. Freeze at -80°C. Samples could be frozen at -25 °C for up to 24 months if needed.

5.5 Methods for 16S rRNA metatoxonomic sequencing

5.5.1 Metataxonomic 16s rRNA sequencing theory.

Each living cell contains nucleic acids which are units for storing genetic information. All prokaryotes have the 16s rRNA gene which consists of hypervariable regions and persevered areas (Baker, Smith and Cowan, 2003). The hypervariable regions are thought to be unique to each species and are used as a genetic marker to identify a species. rRNA sequencing involves several steps as previously described (Mullish *et al.*, 2019a).

1.DNA extraction by mechanical and chemical cell lysis and then buffers are added, then it is washed and bound to a matrix.

2.rRNA amplification: PCR primers bind to the conserved portions that border the hypervariable regions of the rRNA gene (Van de Peer, Chapelle and De Wachter, 1996). Then the 16S rRNA gene is amplified.

3.The amplified DNA is purified and then a second PCR reaction occurs which adds a unique combination of barcoded indices to each sample (Illumina, n.d.). Amplified sequences are purified, denatured, and sequenced.

4.rRNA sequencing: sequencing tools are used to sequence hundreds of base pair paired end reads and they are matched to known samples from a reference database and taxonomic assignments are made.

One limitation of 16s gene sequencing is that different studies use different kits and therefore there may be minor differences between studies. There needs to be a minimum amount of sample to yield enough DNA from the process. Also, there can be contamination of the sample, therefore in the sample processing and aliquoting, it is essential that sterile pipettes and equipment is used.

5.5.2 Methods for human studies

5.5.2.1 DNA extraction

Samples were kept frozen until DNA extraction. Approximately 250mg of stoma effluent was mixed with 0.1mm beads and buffer and placed inside a Bullet Blender Storm (Chembio Ltd, St Albans, UK) and homogenised at speed 8. This step is important for mechanical cell disruption which is especially important for the detection of the genetic material of gram-positive bacteria. Then a commercial DNA extraction kit (PowerLyzer PowerSoil DNA Isolation Kit, Mo Bio, Carlsbad, CA, USA) was used according to manufacturer's instructions as previously described (Taylor *et al.*, 2020). Extracted DNA was then quantified, aliquoted and placed back into storage in a -80^{o}C freezer.

5.5.2.2 16S rRNA sequencing

Sample libraries were constructed using the 16S Metagenomic Sequencing Library Preparation Protocol by Illumina which uses specific V1/V2 hypervariable region primers (Mullish *et al.*, 2018). The Illumina MiSeq platform (Illumina) and MiSeq Reagent Kit v3 was used to perform pooled sample library sequencing (Illumina Inc, Saffron Walden, UK. The rRNA sequence data were analysed using the DADA2 pipeline (v1.18) as previously described(Callahan *et al.*, 2016), using the SILVA bacterial database Volume 138 (https://www.arb-silva.de/ (accessed on 28th July 2020)).

5.5.3 Statistical analysis of 16s rRNA results

5.5.3.1 Alpha diversity

The *alpha diversity* is the variation in the microbes within a sample and can be described using the Shannon index which is a combination of the richness (the number of different species) and its evenness (the distribution of abundances). A high Shannon index means there are a lot of species with equal abundances and a lower Shannon index means the sample is dominated by one

bacterium. Chao1 is a measurement of diversity using abundance data. Chao richness describes the total number of bacterial taxa observed)

As previously described (Taylor *et al.*, 2020) (Mullish *et al.*, 2019b), diversity was determined using Mothur and a combination of R packages including Phyloseq (McMurdie and Holmes, 2013), Vegan, and ggplot2. Statistical tests were performed using GraphPad Prism v7.03. A *p*-value and *q*- value of 0.05 each were considered significant.

5.5.3.2 Beta diversity

The *beta diversity* is the difference in the microbial community in different samples; the Bray-Curtis dissimilarity is based on the difference in abundance where 0 means both samples have the same species at the same abundances and 1 means the samples differ in their species abundances. The UniFrac dissimilarity compares the microbial community between patients by looking at the fraction of branch length that is shared by 2 samples; weighed UniFrac is where the branch lengths are weighted by relative abundances whereas unweighted UniFrac does not include abundance data. Aitchison's distance(Aitchison, 1982) was used for beta-diversity analyses after center log-ratio data transformation (CLR). Weighted UniFrac distance was compared between groups using non-metric multidimensional scaling (NMDS) and permutational analysis of variance (PERMANOVA), using the UniFrac weighted distance matric performed using Mothur, and the R packages vegan and ggplot 2.

The ASV table produced by DADA2 was formatted by grouping rare variants followed by subsampling data to the level of that with the lowest summed read count. No samples were identified as an outlier for summed read count using GraphPad Prism (ROUT, $Q = 1$). STAMP(Parks *et al.*, 2014) analysis was performed to determine the difference in relative abundance between different groups at different levels (phylum, family, genera).

Two group comparison was performed in STAMP using two-sided White's non-parametric t test with Benjamini-Hochberg correction for multiple testing ($q < 0.05$ was considered significant). Those where the difference in the mean proportion of sequences was greater than 1% were selected as significant. ASVs were trimmed if not present in at least 15% of samples. Rare taxa that were present in less than 10% of the other samples were removed as they may be a product of sequencing errors.

5.6 Methods for ^{1}H-NMR analysis

5.6.1 ^{1}H-NMR concepts

Nuclear magnetic resonance (NMR) spectroscopy is a technique that allows a wide range of metabolites in a biofluid to be characterised and measured by the way they interact with an applied radiofrequency energy. Each metabolite within a biofluid has atoms with a nucleus in the centre. The atomic nucleus contains the protons and neutrons and acts like a magnet which can align with the magnetic field. It is the protons which are detected by NMR.

A sample is placed into the probe in the centre of the superconducting magnet where the magnetic field is the strongest and a radiofrequency pulse is applied which excites all the nuclei and makes them spin at a frequency which is proportional to the strength of the magnetic field. The electron density of the molecule also affects the spin (this is known as shielding). The frequency of the spins can be measured by the current they induce in the coil. Each nucleus of a molecule has a different signal. The nuclear spin can be quantified by a quantic number (/) which depends on the reference isotope. Nuclei with a *I* which is not equal to zero can be detected by NMR spectroscopy. Changes in the orientation of nuclear spins are detected as magnetic resonance. When radiofrequency energy is applied to the fluid in a magnetic field of a specific strength, the protons absorb energy and then re-emit it which produces a signal called the free induction decay (FID) which is detected. The FID is converted into an NMR signal by considering the intensity of the magnetic field to calculate the chemical shift. The chemical shift is the effect of the molecule's chemical make up on the resonance frequency. A molecule with 3 different nuclei will have 3 peaks or 3 chemical shifts. The zero point of the chemical shift is set as that of a reference chemical, for example, tetramethylsilane (TMS) or sodium trimethylsilylpropanesulfonate (TSP).

Therefore, each biofluid has a unique NMR signal which correlates to a unique resonance frequency. Each metabolite produces a pattern of peaks depending on the electronic environment surrounding each nucleus. The biofluid signals are compared to that of reference chemicals. When interpreting NMR signal, 'noise' which is peaks without any meaningful signals must be accounted for. Distorted baselines can also alter the intensity of spectra therefore a technique known as baseline estimation and its subtraction is used to correct this. Another challenge is correcting peak shifts which occur due to changes in pH, ion levels, instrument changes and temperature changes. To help overcome this, buffer solutions are used as well as other methods. Data of different samples are then scaled and normalised so that samples can be compared to each other to minimise the effect of variable

dilution of the samples. JRES plots the chemical shift against the proton-proton coupling and helps identify the NMR spectra.

The advantages of NMR include its relatively straightforward preparation and high reproducibility. It provides a snapshot of the most abundant molecules. A disadvantage is that it is less sensitive than mass spectrometry.

5.6.2 Sample preparation for ^{1}H-NMR

Stool, urine and serum samples were defrosted at room temperature with the caps of the containers on to avoid evaporation. A spatula was used to mix each sample.

5.6.2.1 Stoma effluent preparation

As previously described (Taylor *et al.*, 2020), each sample was homogenised with water grade (Fisher Chemical) with 1mg of wet weight of faecal sample in a ratio with 2µL of water and then underwent 5 minutes of vortex and then 20 minutes of centrifugation at 18 000***g*** at 4°C. 540 µL of the supernatant and 60 µL of 1.5 M K O_4 buffer (pH 7.4, 100% of deuterium oxide (D_2O), 2 mM sodium azide and 1% of TSP (3-trimethylsilyl-[2,2,3,3,-$^{2}H_4$]-propionic acid sodium salt) were mixed (Taylor *et al.*, 2020) and the resulting mixture was centrifuged at 18 000 ***g*** at 4°C for 1 min. 580 µL of the supernatant was transferred into a 5 mm NMR tube.

5.6.2.2 Serum preparation

Serum samples were thawed and centrifuged at 18 000 g at 4 ºC for 10 min. 350 µL of the supernatant was mixed with 350 µL of buffer containing 75 mM Na_2HPO_4 (pH 7.4, LC-MS water/D_2O (80:20)), 2 mM sodium azide and 0.08% w/v of TSP (3-trimethylsilyl-[2,2,3,3-$^{2}H_4$]-propionic acid sodium salt). 600 µL of the mixture was transferred to a 5mm NMR tube.

5.6.2.3 Urine preparation

Each urine sample was thawed at room temperature and centrifuged at 18 000 g at 4 ºC for 5 min. 630 µL of the supernatant were mixed with 70 µL of 1.5 M KH_2PO_4 buffer (as above). An aliquot of 600 µL of the mixture was transferred to a 5mm NMR tube.

5.6.3 ^{1}H NMR Spectral analysis

As previously described (Taylor *et al.*, 2020), ^{1}H NMR spectroscopy were acquired at 300 K (urine and stoma effluent), and 310 K (serum) on a Bruker 600 MHz Avance III HD spectrometer. Standard 1-dimensional (1D) pulse sequence was used to obtain the spectra; saturation of the water resonance (noesygppr1d pulse program) during both the relaxation delay (RD = 4s) and mixing time (t_m = 10 ms) were used. The two magnetic field z-gradients were applied for 1 ms. For all analyses, the gain on the receiver was programmed to 90.5 and acquisition time (ACQ) to 2.73s. 1D ^{1}H-NMR spectra were acquired using dummy scans, 64 K data points and with a spectral window of 20 ppm. Each FID was multiplied by an exponential function corresponding to a line broadening of 0.3 Hz (Taylor *et al.*, 2020).

For stoma effluent and urine spectra, 1D ^{1}H-NMR spectra were phased as previously described (Taylor *et al.*, 2020), with baseline correction and calibration to TSP peak at δ^{1}H 0 ppm, and then they were digitised over the range δ^{1}H −0.5 to 11 ppm, and imported into MATLAB (2014a, MathWorks). Prior to probabilistic quotient normalisation (Dieterle *et al.*, 2006), spectral regions containing residual water (δ^{1}H 4.69-4.93), TSP (δ^{1}H −0.50 to 0.50) and noise (δ^{1}H 9.40-11.00) were removed. Serum spectra analysis involved, first acquiring the 1D NOESY-presat and then acquiring the one-dimensional CPMG with water saturation using the Carr–Purcell–Meiboom–Gill pulse sequence (cpmgpr1d). The acquisition parameter was set up in the same way as the 1D NOESY-presat. FIDs were multiplied by an exponential function corresponding to a line broadening of 0.3 Hz. 2D ^{1}H − ^{1}H *J*-resolved experiment was also acquired for each sample to detect the *J*-couplings in the second dimension using the pulse program with suppression of the water resonance during the relaxation delay (jresgpprqf). The acquisition parameters included: 8K points with spectral window of 16.7 ppm for f2 and 40 increments with a spectral window of 78 Hz for f1, dummy scans, incremented delay of 3µs, ACQ of 0.41 s and RD of 2 s. The receiver gain was set to 90.5. A sine bell apodization function was applied. Fourier transformation, tilting by 45°, and symmetrisation along f1 was performed.

For lipoprotein analysis, the low-density lipoprotein (LDL) particle size distribution, cholesterol concentration and more than 100 other lipoprotein parameters were determined using the Bruker IVDr Lipoprotein Subclasses Analysis (B.I.LISA) module with the Bruker Avance IVDr 600 MHz system. The plasma ApoB is the sum of the atherogenic lipoproteins (LDL, very-LDL, intermediary density

lipoprotein and lipoprotein (a)] because each of these carries an apoB100 molecule. Around 70 % of the total apolipoproteins in high-density lipoprotein is made up of ApoAI; the plasma content of apoAI represents the total of antiatherogenic particles.

Extraction of serum phosphocholine and *N*-acetylglycoprotein signals was performed separately. In 1D ^{1}H full resolution spectra of serum, it is possible to observe the methyl groups from the quaternary amine of the choline moiety (R-N^{+}–$(CH_3)_3$) within glycerophosphocholines (main class) and/or ceramide phosphocholines (sphingomyelins, sub class), which are normally present in lipoproteins, here referred as Supramolecular Phosphocholine Composites (SPC-1/2). Same integral region with an offset at d 3.20 or 3.21, that corresponds to SPC-1 or SPC-2, respectively, were extracted for additional correlation analyses.

N-acetyl methyl group from acute-phase proteins such as α-1-acid glycoprotein, haptoglobin, α-1-antitrypsin, α-1-antichymotrypsin, and transferrin produce composite *N*-acetyl signals referred as Glyc-A and Glyc-B. The same integral region from these signals with an offset at d 2.03 or 2.07, for Glyc-A or Glyc-B, respectively, were also extracted from 1D ^{1}H full resolution spectra for additional correlation analysis.

5.6.4 Statistical analysis of NMR results

5.6.4.1 Unsupervised analysis

For the NMR datasets, to determine which metabolites were different between groups, an unsupervised model, principal component analysis (PCA) was used. PCA is a method be able to convert a large amount of information into smaller components so that data can be more easily visualised. The significance of the metabolite difference between groups is determined using the non-parametric univariate analysis, Wilcoxon Rank test or linear regression adjusting for covariates.

5.6.4.2 Supervised analysis

Supervised models such as partial least squares (PLS) and orthogonal partial least squares discriminant analysis (OPLS-DA). This is a type of multivariate linear regression model which can be used to find discriminating metabolic features between different groups. OPLS-DA excludes data which is varied due to physiological differences (such as changes due to dietary intake, or medications). Differences in the peaks between groups are highlighted using statistical correlation

spectroscopy for metabolite identification (STOCSY). A stronger a correlation has an R2 value near to 1. In urine there is a lot of overlap from resonances and STOCSY can help determine the true peak. Metabolite identification is performed by determining the multiplicity of peaks and checking against a database. Another method to filter out the overlapping resonances, in order to pick out rare or low intensity signals is called STORM.

Multivariate data analysis was performed on the 1D ^{1}H Carr-Purcell-Meiboom-Gill (CPMG) spectra. Each spectrum was automatically phased, baseline corrected, digitized over the range δ -0.5 to 11 and imported into MATLAB for further processing (2014a, MathWorks, Natick, U.S.A.). The spectra were referenced to the doublet of the anomeric proton signal of □-glucose at δ 5.23 ppm. The spectral regions corresponding to internal standard (δ -0.5 to 0.63), water (δ 4.5 to 4.9) and noise (δ 5.4 to 6.6 and 8.5 to 11.00) were excluded. Prior to multivariate data analysis, the spectra were normalized using the probabilistic quotient method as previously described (Dieterle *et al.*, 2006). The data set from each type of matrix was auto-scaled and modelled using Partial Least Squares Discriminant Analysis (PLS-DA) in a Monte Carlo Cross-Validation (MCCV) framework using Storey-Tibshirani method of correction for multiple testing. Variables with $q < 0.05$ were considered to be significant. Goodness of fit (R^2Y) was calculated using the training data, and the goodness of prediction (Q^2Y) from test data. Metabolite structure was determined using 2D-NMR experiments and Subset Optimization by Reference Matching (STORM) on 1D ^{1}H-NMR data set(Posma *et al.*, 2012). Internal and external databases such as the Human Metabolome Data Base (HMDB; **http://hmdb.ca/)** (Wishart *et al.*, 2018) and/or the Biological Magnetic Resonance Data Bank (BMRB; **http://www.bmrb.wisc.edu**) were used for confirmation of assignments. The Mann Whitney U test was used to compare metabolite levels for Apoproteins and lipoproteins.

5.7 Methods for mass spectrometry

5.7.1 Mass spectrometry theory

Mass spectrometry (MS) is a technique to analyse the chemical structure of a sample by separating molecules based on their mass to charge ratio (*m/z)*. Mass spectrometry analysis can be untargeted or targeted. In the untargeted approach, all detectable metabolites are analysed, results are represented as a 3D contour map. The concentrations cannot be estimated with untargeted metabonomics. With the targeted approach, detected metabolites can be quantified. The compound of interest is matched to the spectra of a library of mass spectra. If a match is not made, then manual or software assisted interpretation can be performed. An advantage of MS is its ability to detect a large number of molecules.

5.7.2 UPLC-MS methods for bile acid quantification

Bile acids were quantified for serum and stool and a series of pooled quality control (QC) samples, using an established targeted ultrahigh-performance liquid chromatography tandem mass spectrometry (UPLC-MS) method as described (Sarafian *et al.*, 2015) which has a high sensitivity and specificity and can quantify many bile acids (Sarafian *et al.*, 2015; Mullish *et al.*, 2018).

5.7.2.1 Sample preparation for targeted UPLC-MS bile acid profiling

Samples were collected and stored as described in section 5.4 and kept frozen until processing. The serum and stool samples were stored in aliquots of 100-500µL to avoid several freeze-thaw cycles. Organic solvents (HPLC grade) used for the sulfation and precipitation and sodium sulfate were obtained from Sigma-Aldrich (Dorset, UK). All mobile phases were prepared with LC-MS grade solvents, formic acid, and ammonium formate from Sigma-Aldrich (Dorset, UK).

Frozen samples were then thawed at 4^{o}C and transferred to 1 mL Eppendorf 96-deepwell plates (Eppendorf) and centrifuged at maximum speed in an Eppendorf 5810 R equipped with an A-2-DWP-AT rotor (3486g) for 15 min. at 4^{0}C. Supernatant (150 µL) was transferred to 0.5 mL Eppendorf 96-deepwell plates, and 300 µL of ice-cold methanol was added to each sample. Plates were heat sealed (Thermo Fisher Scientific, Hertfordshire UK), then vortexed at 1400 rpm for 15 minutes at 4 °C to homogenize it. Then it was incubated at −20 °C for 20 min. All samples were again centrifuged at 4 °C (3486g) for 15 min prior to decanting of 200 µL of supernatant to Eppendorf microplates for heat sealing and subsequent analysis.

As previously described (Sarafian *et al.*, 2015), bile acid analysis was performed by UPLC (Waters Ltd., Elstree, UK) with Xevo TQ-S mass spectrometer for targeted MS (Waters, Manchester, UK). The MS system had an electrospray ionization source performing in negative ion mode (ESI−). The reversed-phase chromatographic method consisted of a mobile phase system, adapted from existing lipid profiling methods,38 paired with a shorter alkyl chain stationary phase (C8) to facilitate both the separation of BA species and the elution of lipidic matrix content. For this purpose, an ACQUITY BEH C8 column (1.7 µm, 100 mm × 2.1 mm) was selected and used at an operating temperature of 60 °C. The mobile phase solvent A consisted of a volumetric preparation of 100 mL of acetonitrile added to 1 L of ultrapure water, with a final additive concentration of 1 mM ammonium acetate and pH adjusted to 4.15 with acetic acid. Mobile phase solvent B consisted of a volumetric preparation of acetonitrile and 2-propanol in a 1:1 mixture. Critically, the high organic wash step was adjusted in length for the complete elution of observable phospholipids and triglycerides, precluding their accumulation on the column. 5 µL of the samples were injected. To minimize injector carry-over, 3 wash cycles of weak (H2O/ 2-propanol, 90:10) and strong (2-propanol) solvent preparations were performed simultaneously with sample analysis. Mass spectrometry parameters were set at: capillary voltage at 1.5 kV, cone voltage at 60 V, source temperature at 150 °C, desolvation temperature at 600 °C, desolvation gas flow at 1000 L/h, and cone gas flow at 150 L/h. Bile acid species yielding characteristic fragments when subjected to collision-induced dissociation were assayed using multiple reaction monitoring (MRM), while those that did not fragment were assayed by selected ion monitoring (SIM). The transitions for each of the BA standards and deuterated internal standards are provided in Tables S-1 and S-2.

Optimization of Bile Acid Sulfation. LCA, being one of the BAs with the highest concentrations, was selected for the sulfation procedure optimisation. Triplicate samples of lithocholic acid (LCA) (pure reference material) were prepared for 16 sulfation reaction conditions. Sulfur trioxide−pyridine complex was resuspended in CHCl3 (5 mg/mL) or pyridine and added to 20 µL of BA standards (0.5 mg/mL), either with or without sodium sulfate. The reaction was stopped after 1 or 24 h at RT or 55 °C by evaporation to dryness. All samples were stored at −80 °C. Prior to use in analysis, all samples were solubilized in H2O/ acetonitrile/2-propanol (10:6:5, v/v/v).

Purification of Sulphated Bile Acids. Following synthesis, sulphated BAs were detected by UPLC-MS/MS using the chromatographic method described above and multiple reaction monitoring for the $[HSO_4]$ − m/z 97 moiety. Purification of sulphated BAs was performed by tailored chromatographic separations of the reaction mixture using an Acquity UPLC with an XBridge BEH C8 column (3.5 µm, 4.6 mm × 150 mm) (Sarafian *et al.*, 2015),. The solvents used were the same as those described above for the analytical method. The chromatographic separation of each

sulphated BA was repeated a number of times, and fractions were repeatedly collected using a Fraction Collector III (Waters, Manchester, UK) in order to amass sufficient material for subsequent experiments.

Optimization of MRM Transitions and SIR. Source parameters including collision energy and capillary voltage were optimized for each standard by direct infusion combined with the UPLC flow rate and appropriate solvents (e.g., UPLC-MS/ MS conditions). Source parameters were optimized for glycine, taurine, and sulphate fragmentations. These optimizations were automatically performed using Waters IntelliStart software (Waters) and then improved manually. Cone voltage was set to 60 V for all BAs as no significant variation in ionization during the optimization was observed between 10 and 60 V(Sarafian *et al.*, 2015).

5.7.3 Method for profiling short chain fatty acids (UPLC-MS/MS)

A LC-MS method which has been optimised for quantifying SCFAs (Valdivia-Garcia *et al.*, 2022) was used because it has advantages over GC-MS including being faster. Although GC-MS also has a high sensitivity and specificity for SCFA (Mills, Walker and Mughal, 1999).

5.7.3.1 Sample preparation for SCFA

Stool, urine and serum samples were collected from consented participants with Crohn's disease and intestinal failure as described in section 5.3 and 5.4 and kept frozen until processing.

5.7.3.1.1 Stoma effluent extraction

SCFAs were quantified for serum and stool and a series of pooled quality control (QC) samples, using an optimised method as described (Valdivia-Garcia *et al.*, 2022). Samples were collected and stored as described in section 5.3 and 5.4. The serum and stool samples were stored in aliquots of 100-500µL to avoid repeated freeze-thaw cycles. As described (Valdivia-Garcia *et al.*, 2022), stoma effluent were defrosted at room temperature and then mixed with a spatula prior to transferring 10-50mg to a 2ml cryovial with around 100 mg of glass beads (0.5-1mm diameter) (BioSpec Products, Bartlesville, Oklahoma, USA). We recorded the initial wet weight (W_o) of each sample. One milliliter of cold isopropanol (Optima LCMS grade, Fisher, Leicester, UK) was added to the vial and bead beated for three cycles at 6000 rpm for 30 seconds per cycle to homogenize it (Precellys, Bertin Technologies, Montigny Le Bretonneux, France). 300 µL aliquot of homogenized sample was transferred to a pre-weighted (recorded as VW_o) 1.5 mL polypropylene microcentrifuge tube and dried using a vacuum drier (Eppendorf Concentrator Plus, Hamburg, Germany) at 60ºC for 2 hours in order to assess the water percentage in the samples. Following the dry process, the weight of the

microcentrifuge tube containing dry samples was recorded (VW_f). Dry weight (DW) of the samples was calculated.

The remaining homogenized sample in isopropanol (approximately 700 µL) was centrifuged at 16,000 g for 15 minutes at 4ºC (Eppendorf 5430R Centrifuge, Hamburg Germany). The supernatant (was stored at -80ºC in a sterile cryovial. Prior to derivatisation, 45 µL water (LCMS Optima grade, Fisher, Leicester, UK) was mixed with a 5µL aliquot of SE (ten-fold dilution) in a 1 mL, 96 deep well round bottom plate (Eppendorf, Hamburg, Germany) which was covered with a silicone mat (Thermo Scientific Web Seal Massachusetts, USA) and vortexed (Mix Mate Eppendorf, Hamburg, Germany) for 10 seconds at 1400 rpm and stored at 4ºC until derivatisation step.

5.7.3.1.2 Serum extraction

As described (Valdivia-Garcia *et al.*, 2022), samples were defrosted at room temperature maintaining containers capped to avoid evaporation of SCFA. After a brief vortexing, 50 µL of urine or 25 µL of serum was aliquoted to a 1000 µL 96 deep well round bottom plate (Eppendorf, Hamburg, Germany) and 50 µL cold isopropanol (LCMS Optima grade, Fisher, Leicester, UK) was added, resulting in a two-fold (urine) and three-fold (serum) dilution. Silicone mat was used to cover the plate (Thermo Scientific Web Seal Massachusetts, USA) and the plate was vortexed (Mix Mate Eppendorf, Hamburg, Germany) for 10 seconds at 1400 rpm. The plates were centrifuged for 15 minutes at 16,000 g at 4ºC (Eppendorf 5430R, Hamburg, Germany) and the supernatant added to new plates, which were covered with a silicone mat and stored at -80°C until the derivatization.

5.7.3.1.3 Preparation of standard solutions

As described (Valdivia-Garcia *et al.*, 2022) acids with 98-99% purity were purchased from Sigma-Aldrich (Steinheim, Germany) including: lactic (C2), acetic (C2), propionic (C3), butyric (C4), isobutyric (C4), 2-hydroxybutyric (C4), isovaleric (C5), 2-methylbutyric (C5), valeric (C5) and hexanoic (C6). Isotope-labelled internal standards were purchased from Sigma-Aldrich (St. Louis, MO). Isobutyric acid-D6 was purchased from Molecular Dimensions (Newmarket, USA). Short chain carboxylic acids (SCCAs) stock solutions at 1 M concentration were prepared in a mixture of deionized water and isopropanol (Optima LCMS grade, Fisher, Leicester, UK) at 1:1 v/v, and stored at 4ºC in silanized glass vials. 10 concentrations (100, 50, 25, 10, 5, 2.5, 1, 0.5, 0.25 and 0 µM) were produced by serial dilutions in water from 1 M stock solutions to establish calibration curves. Labelled internal standards were diluted from stock solutions at 1 M to obtain final concentrations of 60 µM for acetic, lactic and propionic acids (Internal

Solution A); and 6 µM for isobutyric, butyric, isovaleric, valeric and hexanoic acids (Internal Solution B).

As described (Valdivia-Garcia *et al.*, 2022), for standard calibration curve solutions for stool and stoma samples, 5 µL of SE was added to 45 µL water in a 1 mL 96 deep well plate. 25 µL labelled internal standard solution A,= 25 µL labelled internal standard solution B, 20 µL of 200 mM 3-nitrophenylhydrazine hydrochloride (3-NPH; Sigma Aldrich, Steinheim, Germany; prepared in 50% v/v acetonitrile in water), and 20 µL of 120 mM *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC) prepared in 6% pyridine in ethanol (v/v) solution (Sigma Aldrich, Steinheim, Germany) were added to each well.

The plate was covered with a silicone mat and mixed at 1400 rpm for 10 seconds and then placed on a heat block (Digital Dry Block Heater, Thermo Scientific, Massachusetts, USA) for 30 minutes at 40°C. Then the plate was removed from the heat black and 200 µL 0.1% formic solution in deionized water was added and mixed for 10 seconds at 1400 rpm and centrifuged at 4000 g for 5 minutes at 4°C to quench the derivatization. 200 µL of the supernatant was transferred to a 300-µL round bottom 96 well plate (Eppendorf, Hamburg, Germany) and an aluminum cover (Corning, ME, USA) was used to cover it and placed at 120°C for 12 seconds (ALPS V50, Thermo Scientific, Massachusetts, USA), and stored at 4°C until analysis.

UPLC-MS

A reverse phase (RP) chromatographic separation was performed on a Waters Acquity Binary Management (BSM) UHPLC system (Milford, USA) as described (Valdivia-Garcia *et al.*, 2022) data was MassLynx V4.2 software (Waters, Milford, USA) was used for MS data and TargetLynx XS software (Waters, Milford, USA) was used to process MS data. The MS capillary voltage was set at 2000 V; cone voltage, 60 V; cone temperature, 150°C; desolvation gas flow, 1000 L/h; and desolvation temperature, 500°C. ChemDraw Pro (Version 19.01.08, Perking Elmer Informatics Inc, Waltham, MA, USA) was used to verify ion pair masses (Q1>Q3) and fragmentation patterns.

Concentrations of SCFAs were calculated taking into account the dilution factor (Df), for example three-fold for serum and ten-fold for fecal samples.

The lowest limit of detection (LLOQ) was set at a SCCA signal to noise ratio ≥10. The limit of quantitation (LOQ) for all SCCAs was at 3.3 times the L vb (s/n) ≥ 30 following recommended values for chromatographic validation of metabolites (Lister, 2005; Panuwet *et al.*, 2016).

5.7.4 Statistical analysis of mass spectrometry results

Discovering significant differences or correlations in microbiota or metabolites correlations involves parametric or non-parametric univariate and multivariate analysis. However, given that there are many variables, a model called partial least squares is used to correlate the microbiome and the metabolome.

For all biofluids, when the results of more than 20% of samples returned as less than the lower limit of quantification (<LLOQ), or greater than the upper limit of quantification (>ULOQ), metabolites were not analysed.

Univariate analysis with non-parametric Mann Whitney U test was performed to establish significant BA alterations between the groups.

Principal component analysis (PCA) was carried out on BA peaks with log transformation and mean centering using metaboanalyst v5. OPLS- DA was performed using metaboanalyst v5.

Chapter 6: Microbiota and metabonomic changes in patients with Crohn's disease and intestinal failure.

6.1 Introduction

The microbiota and metabonomic profile of patients with Crohn's disease have been well studied as described in **Section 4.1** and **4.3** respectively. The changes seen in intestinal failure are described in **Section 4.2** and **Section 4.4**. However, multiomics in patients with CD-IF have been less well studied, with only 4 CD-IF patients in the largest study (Table 11). Multiomics gives insight into host-microbiota interactions. Small bowel resection affects the luminal environment; there is an increased transit time, a higher oxygen environment and reduced surface area for reabsorption of nutrients and bile acids; these changes impact the metabonome, as well as the microbiome. 16s rRNA metataxonomic sequencing was used to detect any changes in the microbiota between patients with different bowel length and disease activity status. MS and NMR are complimentary to each other and were used to measure differences in metabolites between different groups.

6.2 Aims

- Determine differences in the microbiota and metabonome of patients with CD-IF comparing different small bowel lengths <150cm vs ≥150cm
- Determine differences in the microbiota and metabonome of patients with CD-IF comparing those with active disease and those in remission
- Determine differences in the microbiota and metabonome of patients with CD-IF comparing those with an end enterostomy and those with colon in continuity

6.3 Methods

Detailed methods for participant recruitment are described in **Section 5.2**. Methods for sample collection is described in **Section 5.3** and **5.4** and methods for gut microbiota profiling, NMR and LCMS are described in **Section 5.5, 5.6** and **5.7** respectively.

Remission was defined as an absence of any inflammation endoscopically and/or radiologically, with an faecal calprotectin <100 μg/g. HBI and CDAI scores were not used as patients with IF often have a stoma so cannot report stool frequency, and they may have other reasons for the abdominal pain or reduced general well-being. SES-CD and CDEIS scores were not used because these are segmental scores and our patients have altered anatomy due to surgery.

Statistical analysis for clinical features were undertaken using the chi squared test for categorical data, and the Mann-Whitney test for non-parametric continuous data. Univariate logical regression was used for variables. For metabolite comparison, statistical analysis is described in **Section 5.6.4** and **Section 5.7.** Univariate analysis was performed using Mann Whitney test to compare metabolites between groups. For multivariate analysis PCA and OPLS-DA were used for metabolites. Alpha and Beta diversity were calculated using R package and graph pad prism 9.0.

The bowel length was available for all patients with CD-IF since this is determined in all patients referred, either from the operation note, or where this is not available, imaging studies are performed, or existing imaging is reviewed to determine the small bowel length.

6.4 Results - Clinical details

6.4.1 Clinical details of all participants

The clinical details of 32 patients are shown (Table 13). Of the 32 participants, 20 provided stoma effluent 27 provided serum, and 28 provided urine; the clinical details of the participants for each group are shown (Table 14). The median age was 54 years, 20 (63%) were female and 81% were caucasian and the median age at diagnosis of CD was 21 years and the median age at diagnosis of IF was 45.5 years. Patients had IF for 1-27 years and had a mean weight of 65.1kg. The mean small bowel length was 106.1cm. 78% of patients had an enterostomy and 22% had small bowel anastomosed to part of a colon in continuity (CIC). 81% required home parenteral nutrition (HPN) and 19% required home IV fluids (HIVF).

85% were in remission for the previous 1 year, 22% were smokers, 28% had perianal disease and 3% had upper GI disease. 7 patients had isolated ileal disease (L1) and 25 had ileocolonic disease (L3). 19% were currently on biologics for 2-13 years: 1 was receiving vedolizumab, 1 ustekinumab and 4 adalimumab. The mean CRP was 4.4 mg/dl. 25 patients had bowel length <150cm and 7 had bowel length $\geq$150cm. 25 patients had small bowel ending in a stoma (ileostomy or jejunostomy) and 7 patients had small bowel anastomosed to the colon (of which 3 had a colostomy). Of the 7 patients with a surgical anastomosis to colon in continuity (CIC): 5 patients had more than >50% of their colon remaining and 2 patient had <50% of their colon remaining.

Table 13 Clinical details of study participants

Total	32 patients
Age median (IQR), years	54 (46.8, 64.3)
Gender n, (%)	20 female 12 male
Age at diagnosis of Crohn's disease median (IQR), years	21 (15.0, 35.5)
Duration of Crohn's disease median (IQR), years	32 (18, 38.3)
Duration of Crohn's disease until Intestinal failure median (IQR), years	22.0 (9.8, 26.0)
Age at diagnosis of Intestinal Failure, median (IQR), years	45.5 (36.5, 57.0)
Duration of Intestinal Failure, median (IQR), years	7.5 (4, 11.5)
Ethnicity	26 caucasian 6 non-caucasian
Duration since last surgery median (IQR), years	8.5 (1-34)
Weight mean ± SD (range), kg	65.1 ± 15.3 (40.2-113)
BMI mean ± SD (range), kg/m^2	23.7± 5.2 (16.6-43.1)
Bowel length mean ± SD (range), cm	106.1 ±44.9 (15-210)
Enterostomy vs continuity with colon	Enterostomy 25 CIC 7
Remaining colon	none 25 <50% 2 >50% 5
Number of small bowel resections mean (range)	2.9 (1-7)
Number of small bowel resections	1 – 3 2 -14 3 - 6 4 - 3 5 - 5 6 - 1
Parenteral nutrition (PN) or fluids (HIVF)	HPN 26 HIVF 6
Disease activity 1 year	Remission 25 Active 7
Smoking	Yes 7 No 25
Perianal disease	Yes 9 No 23
Upper gastrointestinal disease	Yes 1 No 31

Extra intestinal manifestations	Yes 3 No 29
Biologics prior to CD-IF diagnosis	Yes 6 No 26 1 Vedolizumab 1 Ustekinumab 4 Adalimumab
Duration of biologics median (range), y	8 (2-13)
Disease location at diagnosis	L1 7 L2 1 L3 24
Disease location at follow up	L1 7 L0 L3 25
Disease Behaviour at diagnosis	B1 14 B2 8 B3 10
Disease Behaviour at follow up	B1 14 B2 7 B3 11
C-reactive protein mean ± SD (range), (mg/dl)	4.4 ± 4.1 (0.6- 18.0)

Table 14 Clinical data of study participants with depending on sample type submitted.

Total	Stoma effluent (n=20)	Serum (n=27)	Urine (n=28)
Age median, years	54	54	55
Gender n, (%)	12F 8M	19F 8M	16F 12M
Ethnicity	16 caucasian 4 asian	23 caucasian 4 asian	23 caucasian 4 asian
Age at diagnosis of Crohn's disease median, years	21	21	21.5
Duration of Crohn's disease median, years	31.5	32	31.5
Age at diagnosis of Intestinal failure, median (IQR), years	45	46	47
Duration of Intestinal Failure, median, years	9	6	8
Duration of Crohn's disease to intestinal failure, median, years	23	22	22
Weight mean, kg	63.1	60.7	60.6
BMI mean ± SD (range), kg/m^2	23.7	23.1	23.1
Bowel length mean ± SD (range), cm	108.9	106.9	101.8

Enterostomy vs continuity with colon	Enterostomy 20 CIC 0	Enterostomy 20 CIC 7	Enterostomy 20 CIC 7
Parenteral nutrition (PN) or fluids (HIVF)	HPN 15 HIVF 5	HPN 22 HIVF 5	HPN 23 HIVF 5
Disease activity 1 year	Remission 18 Active 2	Remission 22 Active 6	Remission 22 Active 6
Smoking	Yes 6 No 14	Yes 5 No 23	Yes 5 No 22
Perianal disease	Yes 4 No 16	Yes 7 No 20	Yes 6 No 23
Upper gastrointestinal disease	Yes 1 No 19	Yes 1 No 26	Yes 1 No 27
Biologics	Yes 3 No 17	Yes 6 No 21	Yes 6 No 22
C-reactive protein mean (range), (mg/dl)	3.3	4.1	4.2

6.4.2 Comparing clinical features of CD-IF small bowel length <150cm vs ≥150cm

Patients with small bowel length (BL) <150cm (n=25) vs ≥150cm (n=7) had a mean small bowel length (BL) of 88.5cm and 167.1cm respectively. There was no difference in the age, age of CD diagnosis, age of IF diagnosis, duration of CD, duration of IF, gender, ethnicity, number of small bowel resections, disease activity, parenteral nutrition requirement, biologic use or proportion with enterostomy between the two groups. There were more smokers in the ≥150cm group.

Table 15 Comparing clinical features of bowel length <150cm group and >150cm group.

Total	**<150cm small bowel (25)**	**≥150cm small bowel (7)**	***P* value**
Age median (IQR), years	55 (49.0, 65.0)	49 (46.0,58.0)	0.10
Gender n, (%)	14F 11M	6F 1M	0.33
Ethnicity	Caucasian 20	Caucasian 6	0.63

	Non-Caucasian 5	Non-Caucasian 1	
Age at diagnosis of Crohn's disease median (IQR), years	21.5 (16.0, 35.0)	15 (13.0, 36.0)	0.2
Duration of Crohn's disease median (IQR), years	32 (18.0, 39.0)	32 (22.0, 34.5)	0.14
Age at diagnosis of Intestinal failure, median (IQR), years	45.5 (35.0, 61.0)	45 (32.0, 48.0)	0.64
Duration of Intestinal Failure, median (IQR), years	8 (4.0, 13.0)	7(5.0, 10.0)	0.18
Duration of CD until IF median (IQR), years	22 (10.0, 26.0)	24 (11.0, 28.0)	0.77
Time since last surgery median (IQR), years	8.5 (5.5, 15.8)	7 (5.0, 10.0)	0.43
Number of small bowel resections mean	2.9± 1.4	2.4±1.7	0.41
BMI mean ± SD (kg/m^2)	24.0 ± 5.3	22.7 ± 5.4	0.48
Bowel length mean ± SD (cm)	88.5 ± 32.4 (15-140)	167.1 ± 24.3 (150-210)	**<0.001**
Enterostomy vs continuity with colon	Enterostomy 18 CIC 7	Enterostomy 7 CIC 0	0.11
Parenteral nutrition (PN) or fluids (HIVF)	PN 20 HIVF 5	PN 6 HIVF 1	0.63
Disease activity **Remission vs active**	Active 7 Remission 18	Active 0 Remission 7	0.11
Smoking	Yes 3 No 22	Yes 4 No 3	**0.01**
Perianal disease	Yes 6 No 19	Yes 3 No 4	0.16
Upper gastrointestinal disease	Yes 1 No 24	Yes 0 No 7	0.59
Biologics	Yes 5 No 20	Yes 1 No 6	0.21
Disease location at follow up	L1 6 L2 0 L3 19	L1 1 L2 0 L3 6	0.53

Disease behaviour at follow up	B1 9 B2 6 B3 10	B1 5 B2 1 B3 1	0.16
C-reactive protein mean ± SD (range), mg/dl	4.8 ± 4.4 (0.6-18.1)	3.2 ± 3.1 (0.6-9.4)	0.70

6.4.3 Comparing clinical features between active disease vs remission.

25/32 patients were in remission. Given the heterogenous nature of CD, the clinical, endoscopic or radiological findings to categorise the 7 patients with active disease are described:

1. 10cm of neoterminal ileal enhancement on CT and CRP 7.6 mg/dl
2. Active disease on MRI with calprotectin 390μg/g.
3. Mild erosions and inflammation on ileoscopy and a faecal calprotectin of 394μg/g.
4. Histology 2017 obtained at surgery for presumed adhesional obstruction showed active disease.
5. MRI showed a long segment of disease with faecal calprotectin of >2000μg/g.
6. CT abdomen showed inflammation and stricturing.
7. Colonoscopy showed anastomotic inflammation.

When comparing those with active disease (n=7) in the last year vs those in remission (n=25) (Table 16), patients with active disease had a higher mean CRP (9.0mg/dl vs 3.2mg/dl). The main difference was that patients with active disease more often had an anastomosis with colon in continuity (71% vs 8%). L3 disease was more common in the remission group, possibly owing to patients with L3 disease having more colectomy and enterostomy; patients with enterostomy are more likely to be in remission. 3/25 patients in remission had CRP>5 mg/dl which could occur for other reasons such as EIMS or recent infections. Conversely In the active disease group, 1/7 patient had a CRP of 2.1 and had a chronic inflammatory stricture on imaging and a faecal calprotectin of 230μg/g which supports the findings of other studies that the CRP can be normal in active Crohn's disease. There was no difference in the other clinical variables assessed.

Table 16 Comparing clinical features active vs remission for 1 year.

Total	**Remission (25)**	**Active (7)**	***P* value**
Age median (IQR), years	55 (47.0, 64.0)	54(43.5, 60.5)	0.75
Gender n, (%)	17F 8M	3F 4M	0.26
Age at diagnosis of Crohn's disease median (IQR), years	21 (13.0, 35.0)	21 (18.5, 34.5)	0.30
Duration of Crohn's disease median (IQR), years	33 (25-39)	17 (11.5-31)	0.17
Age at diagnosis of Intestinal failure, median (IQR), years	45 (37.0, 57.0)	46 (38.0, 55.7)	0.99
Duration of Intestinal Failure, median (IQR), years	8 (4.0, 13.0)	6 (4.0, 9.5)	0.39
Duration of Crohn's disease until IF, median (IQR), years)	23(14.0, 26.0)	11(5.0, 24.0)	0.28
Time since last surgery median (IQR), years	9(4.0, 17.0)	6(1.0, 10.0)	0.18
Number of small bowel resections mean ± SD	2.7 ± 1.3	2.8 ± 1.9	0.92
BMI mean ± SD (kg/m^2)	24.1 ± 5.6	22.5 ± 3.4	0.70
Bowel length mean ± SD (cm)	111 ± 47	88.6 ± 33.9	0.32
Enterostomy vs colon in continuity (CIC)	Enterostomy 23 CIC 2	Enterostomy 2 CIC 5	**0.001**
Parenteral nutrition (PN) or fluids (HIVF)	HPN 17 HIVF 8	HPN 6 HIVF 1	0.36
Smoking	Yes 7 No 18	Yes 0 No 7	0.11
Perianal disease	Yes 1 No 24	Yes 0 No 7	0.59
Upper gastrointestinal disease	Yes 0 No 22	Yes 1 No 9	0.13

Biologics	Yes 5 No 20	Yes 1 No 6	0.73
Disease location at follow up	L1 4 L2 0 L3 21	L1 3 L2 0 L3 4	0.13
Disease behaviour at follow up	B1 13 B2 3 B3 9	B1 1 B2 4 B3 2	0.07
C-reactive protein mean ± SD (mg/dl)	3.2 ± 2.8	9.0 ± 5.0	**0.004**

6.4.4 Comparing clinical features of patients with enterostomy vs colon in continuity.

Patients with an enterostomy (n=25) were more likely to be in remission compared to those with small bowel surgically anastomosed to a colon in continuity (CIC) (n=7); 92% of the enterostomy group were in remission compared to 29% in the CIC group, p<0.001 (Table 17). The enterostomy group had a lower mean CRP (3.3mg/L vs 8.4mg/L) (Figure 4). Patients in the enterostomy group also had more L3 disease. Patients in the enterostomy group had a longer mean small bowel length (111.6.cm vs 87.5cm) but this did not reach significance. This may be because the enterostomy group had less active disease, however, there was no difference in the mean number of small bowel resections, and we do not have complete information about the length of bowel resected at each surgery. There was no difference in the other clinical variables assessed.

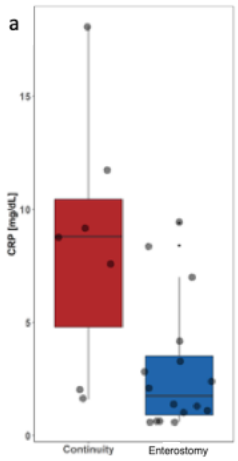
Table 17 Comparing clinical features of patients with enterostomy vs colon in continuity.

Total	**Enterostomy (25)**	**Colon in continuity (7)**	***P* value**
Age median (range), years	56 (49.0, 64.0)	49(42.0, 62.0)	0.10
Gender n, (%)	16F 9M	4F 3M	0.29
Age at diagnosis of Crohn's disease median (range), years	22(15.0, 37.0)	17(16.5, 22.5)	0.99

Duration of Crohn's disease median (range), years	32(24.0, 38.0)	33(15.5, 41.0)	0.95
Age at diagnosis of Intestinal failure, median (range), years	46 (41.0, 57.0)	45(30.0, 55.5)	0.56
Duration of Intestinal Failure, median (range), years	8 (3.0, 13.0)	6(5.5, 11.0)	0.72
Duration of Crohn's disease until IF, median (range), years	22 (10.0, 26.0)	14(10.0, 33.0)	0.68
Number of small bowel resections mean	2.8 ± 1.5	2.6 ± 1.5	0.77
BMI mean ± SD (kg/m^2)	24.3 ± 5.6	21.5 ± 2.7	0.13
Bowel length mean ± SD (cm)	111.6 ± 45.6	85.7 ± 38.8	0.27
Parenteral nutrition (PN) or fluids (HIVF)	PN 19 HIVF 6	PN 7 HIVF 0	0.16
Remission vs active	Active 2 Remission 23	Active 5 Remission 2	**<0.001**
Smoking	Yes 7 No 18	Yes 0 No 7	0.49
Perianal disease	Yes 7 No 18	Yes 2 No 5	0.91
Upper gastrointestinal disease	Yes 1 No 24	Yes 0 No 7	0.59
Biologics	Yes 4 No 21	Yes 2 No 5	0.13
Disease location at follow up	L1 3 L2 0 L3 22	L1 4 L2 0 L3 3	**0.01**
Disease behaviour at follow up	B1 12 B2 4 B3 9	B1 2 B2 3 B3 2	0.42

C-reactive protein mean ± SD (mg/dl)	3.3 ± 2.9	8.4 ± 5.7	**0.02**

Figure 4 CRP in CD-IF with an anastomosis to colon in continuity (red) compared to those with an enterostomy (blue).



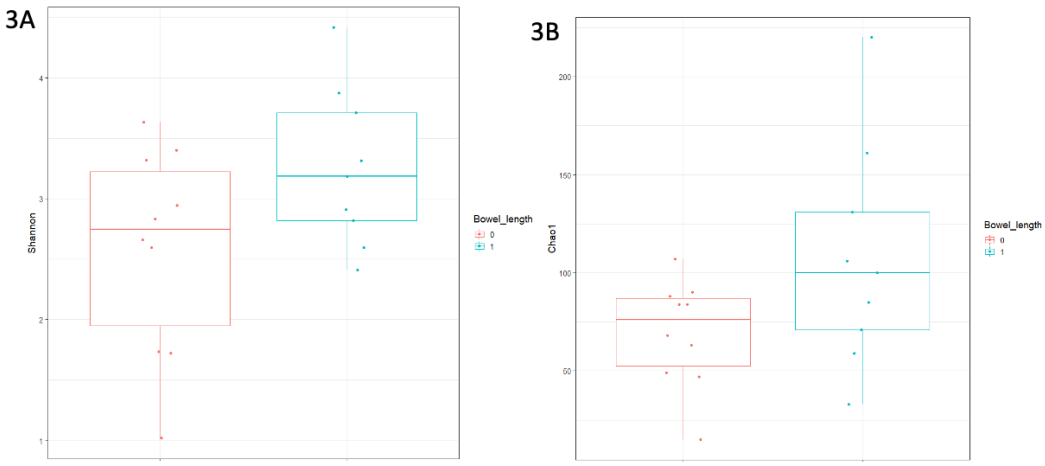
6.5 Results- Gut Microbiota analysis

6.5.1 Alpha Diversity

There was no difference in the alpha diversity (assessed using Shannon diversity index), nor richness (assessed using Chao 1), between patients with bowel length <150cm vs ≥150cm (Figure 4A and Figure 4B). We could not compare patients with active disease vs remission because only 2/18 patient had active disease.

Figure 5 Effect of bowel length on faecal microbial alpha diversity comparing bowel length <150cm vs ≥150cm. A) Shannon diversity index p=0.07, B) Chao1

< 150cm n=11 (red) and >150cm n=7 (blue) p=0.07

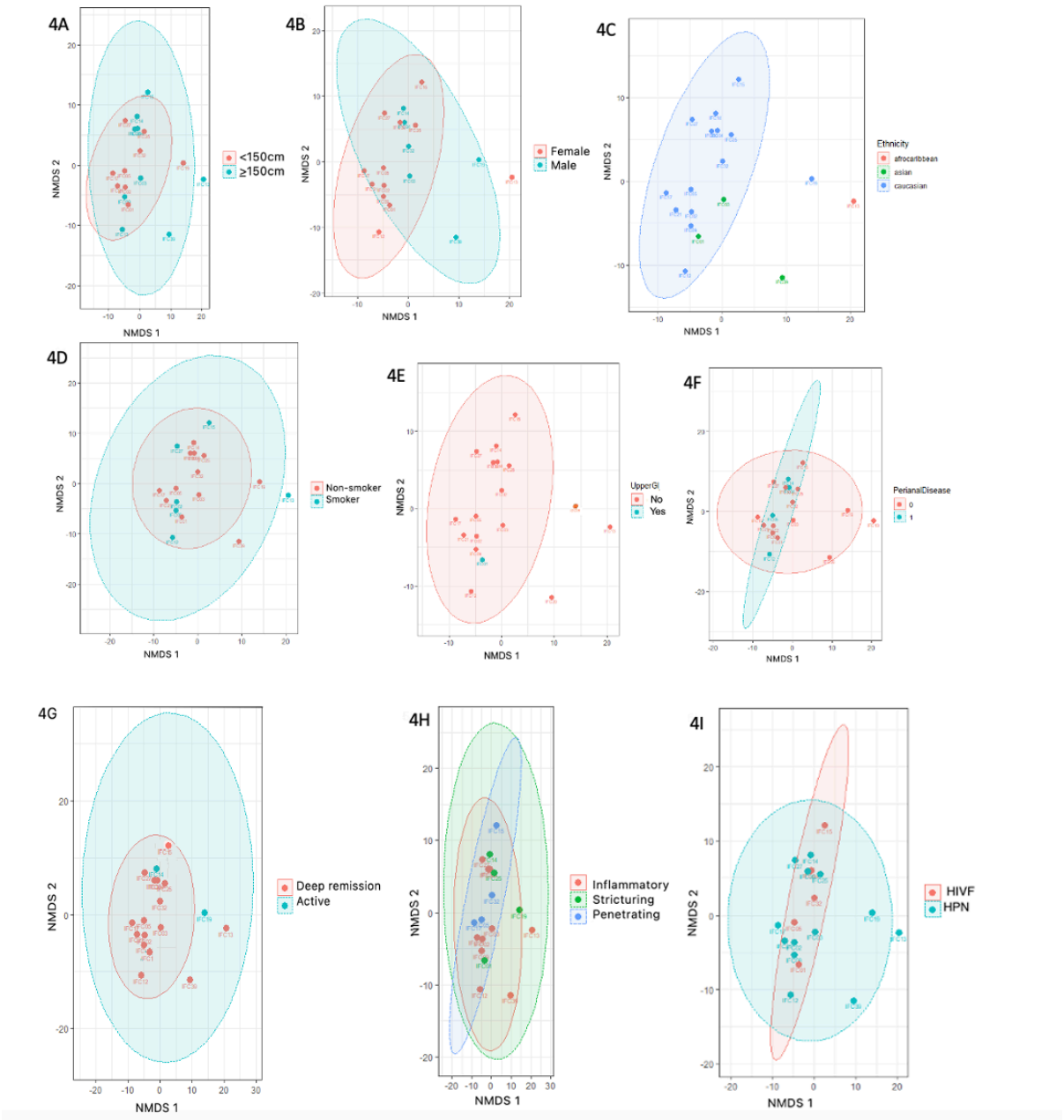


6.5.2 Beta diversity

There was no difference in the stool community structure, assessed using non-metric multidimensional scaling (NMDS) with PERMANOVA. p values were generated using the UniFrac weighted distance matrix (Mothur and R statistical package). Stool samples were inseparable when comparing: <150cm vs≥150cm (Figure 5A), females vs males (Figure 5B), Caucasian vs Asian vs Afro-Caribbean (Figure 5C), non-smokers vs smokers (Figure 5D), the presence of upper GI disease (no/yes)(Figure 5E), the presence of perianal disease (no/yes) (Figure 5F), active vs remission (Figure 5G), disease behaviour (inflammatory vs stricturing vs penetrating) (Figure 5H), HIVF vs HPN (Figure 5I).

Figure 6 non-metric multidimensional scaling plot of gut community structure different groups

Showing weighted UniFrac distance with 95% CI interval ellipses. There was no difference in the weighted UniFrac distance of the following groups with non-significance calculated using PERMANOVA: A) Bowel length<150cm(red) vs ≥150cm (green), B) females (red) vs males (green), C) Caucasian (blue) vs Asian (green) vs Afro-Caribbean (red) ethnicity, D) non-smokers (red) vs smokers (green), E) UGI involvement no (red)/yes(green), F) perianal involvement no (red)/yes(green), G) remission (red) vs active disease 1 year(blue), H)disease behaviour (inflammatory (red) vs structuring (green) vs penetrating (blue), I) home intravenous fluids (red) vs home parenteral nutrition (green).



6.5.3 Stool microbiota abundance results in CD-IF

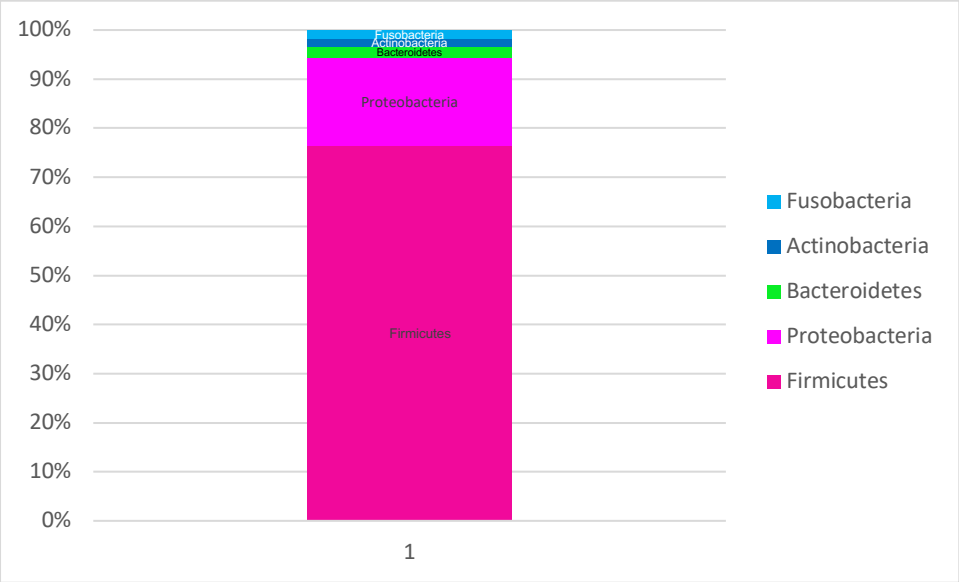
Whilst I did not have a control group, in the discussion **Section 6.9.2,** I discuss how the results in CD-IF compared to patients with an ileostomy without IF, and in healthy controls with an intact colon, in the published literature.

6.5.3.1 Gut microbiota composition in CD-IF cohort

16s rRNA data from stool was analysed at phylum, family, genera, and where possible, species level. Where species level annotation was not possible, higher taxonomic categorisation was performed.

Amongst 18 patients with CD-IF, the most abundant phyla were *Firmicutes* and *Proteobacteria*, with a suppression of *Bacteroidetes* (Figure 6). 4 most prevalent organism at Family level were *Veillonellaceae*, *Lactobacillaceae*, *Streptococcaceae* and *Enterobacteriaceae* (Figure 7B).
The 4 most abundant genera were *Veillonella*, *Lactobacillus*, *Streptococcus*, and *Escherichia* (Figure 7C). The predominance of *Firmicutes* was mainly accounted for by the presence of *Streptococcus*, *Lactobacillus* and *Veillonella*; with these 3 species making up 70% of this phylum. Notably, there was a distinct lack of the anaerobic *Clostridium clusters XIVa* and *Clostridium cluster IV* which includes *Ruminococcus* and *Lachnospira* which were present at very low levels in our samples; these normally predominate in faecal samples in patients with a normal GI tract anatomy in health.
There was a high abundance of *Proteobacteria* which was predominantly made up of 3 families: *Enterobacteriaceae*, *Neisseriaceae*, *Pasteurellaceae*. There was a **low abundance of the *Bacteriodetes*** phylum; this is usually the second most abundant phylum in health.

Figure 7 Relative abundance of gut microbiota at phylum level



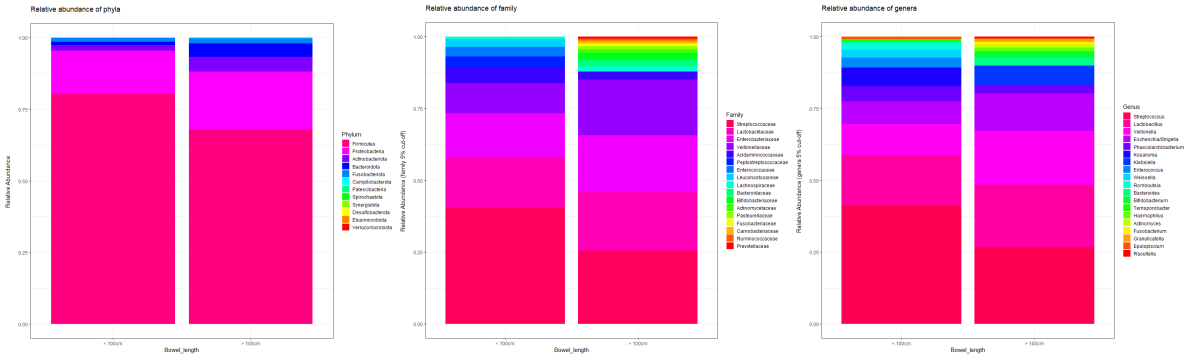
6.5.4 Comparing stool metataxonomic profiles between active disease vs remission.

We could not compare the microbiota of active disease vs remission as only 2/18 patients had active disease.

6.5.5 Comparing stool metataxonomic profiles between small bowel <150cm vs ≥150cm.

There was a similar relative abundance between patients with small bowel length <100cm vs ≥100cm calculated using White's non-parametric T-test with Benjamini-Hochberg FDR correction (Figure 7A-C).

Figure 8 Relative abundance of gut microbiota comparing <100cm vs >100cm A: phyla level; B: family level; C: genera level.



6.5.6 Differential abundance

6.5.6.1. Differential abundance correlating ASVs and clinical variables.

Differential abundance performed on 18 faecal samples did not find correlations between ASVs and any of the following variables: age, gender M/F, BMI, smoker yes/no and active vs remission. The only significant ASV correlating to a variable was that *Streptococcus parasanguinis* increased with increasing continuous CRP (Figure 8A).

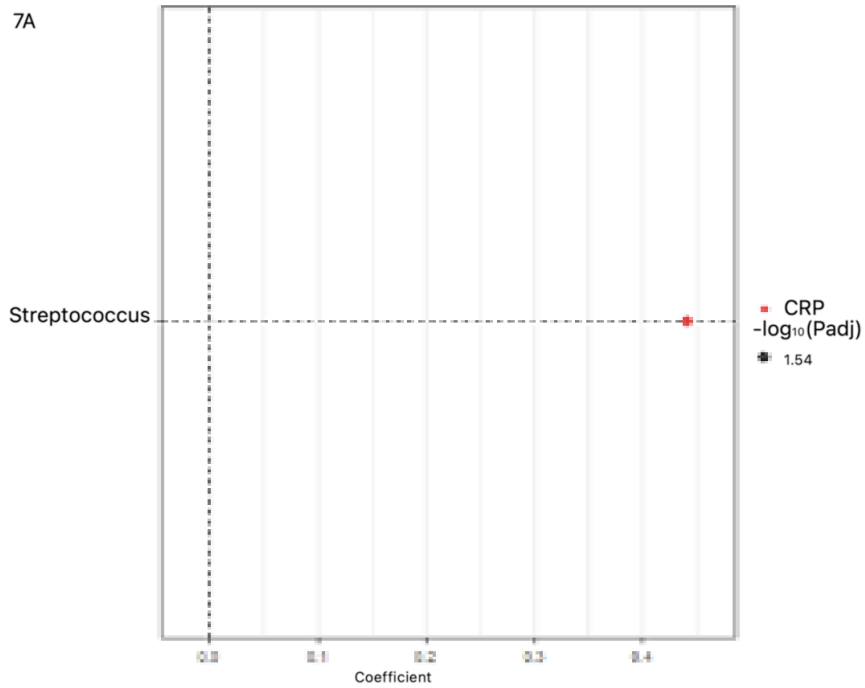
6.5.6.2. Differential abundance correlating ASVs and SCFAs

Differential abundance performed on 13 paired faecal and serum samples showed that the only significant correlations between ASVs and SCFAs were:

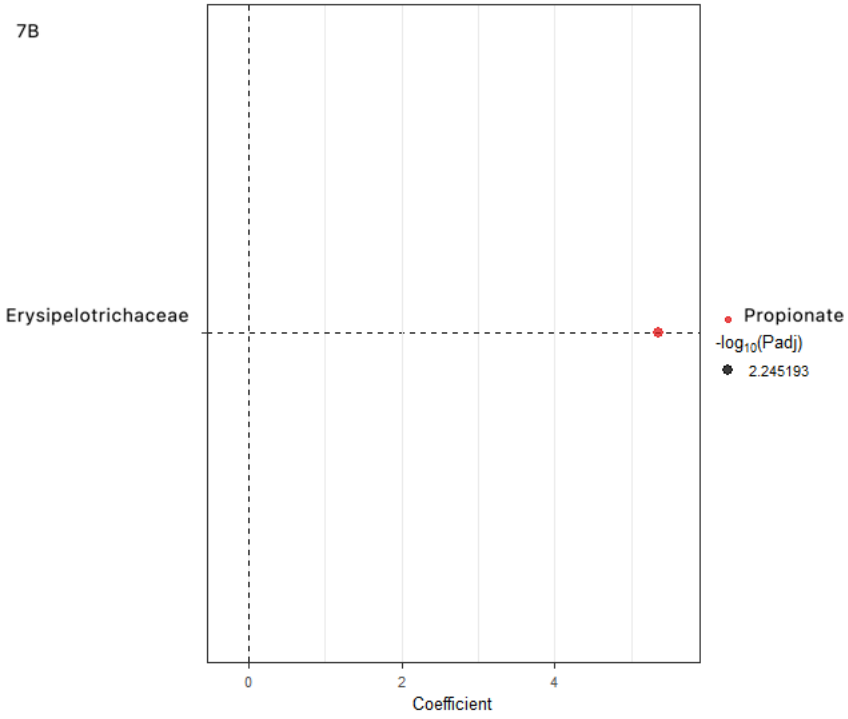
1. *Erysipelotrichceae* correlated positively with propionate (Figure 8B)
2. *Diallister*, *Erysipelotrichceae* and *Alistipes* correlated positively with X2-OH-Butyrate (Figure 8C).

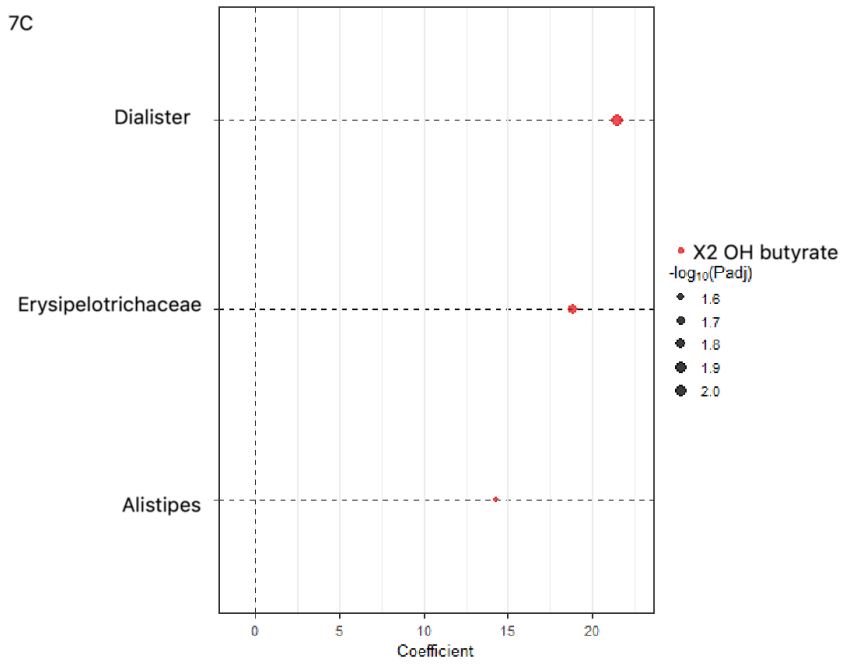
Figure 9 Differential abundance plots correlating amplicon sequence variants to: A) CRP, B) propionate, C) butyrate.

7A


Streptococcus
CRP
-log10(Padj)
1.54
Coefficient

7B


Erysipelotrichaceae
Propionate
-log10(Padj)
2.245193
0
2
4
Coefficient



6.6 Results - Metabonomic profiling of bile acids.

Bile acids were quantified using UPLC-MS. 138 serum bile acids and 69 stool bile acids were analysed (Sarafian *et al.*, 2015), but after exclusion of results where more than 20% of metabolites were lower than the limit of quantification (LLOQ) or above the upper limit of quantification (ULOQ), only 8 serum bile acids and 12 stool bile acids were able to be analysed.

6.6.3 Paired analysis of serum and stoma effluent

Although bile acids were measured in corresponding serum and stool samples from the same patients, only 1, gCDCA-3-sulphate could be compared because the remaining bile acids were mainly below the LLOQ, or above the ULOQ (stool CA and gCDCA).

Stool and serum gCDCA acid -3-sulfate were not significantly correlated (n=15 paired samples, r=0.44, p=0.09, Spearman's correlation). There was no correlation even after adjusting for covariates.

6.6.4 Comparing serum bile acids in active disease vs remission.

6.6.4.1 Multivariate analysis active disease vs remission

On multivariate analysis of serum bile acids, PCA showed separation of active disease vs remission (Figure 9A). Supervised analysis performed with OPLS-DA (Figure 9B) showed separation between active disease and remission, p=0.0008. Model validation is shown in Table 19 and p values were calculated using cross validated ANOVA (CV ANOVA).

Figure 10 PCA score plots comparing serum bile acids in remission (0) and active disease (1).

A) PCA; B) OPLS-DA

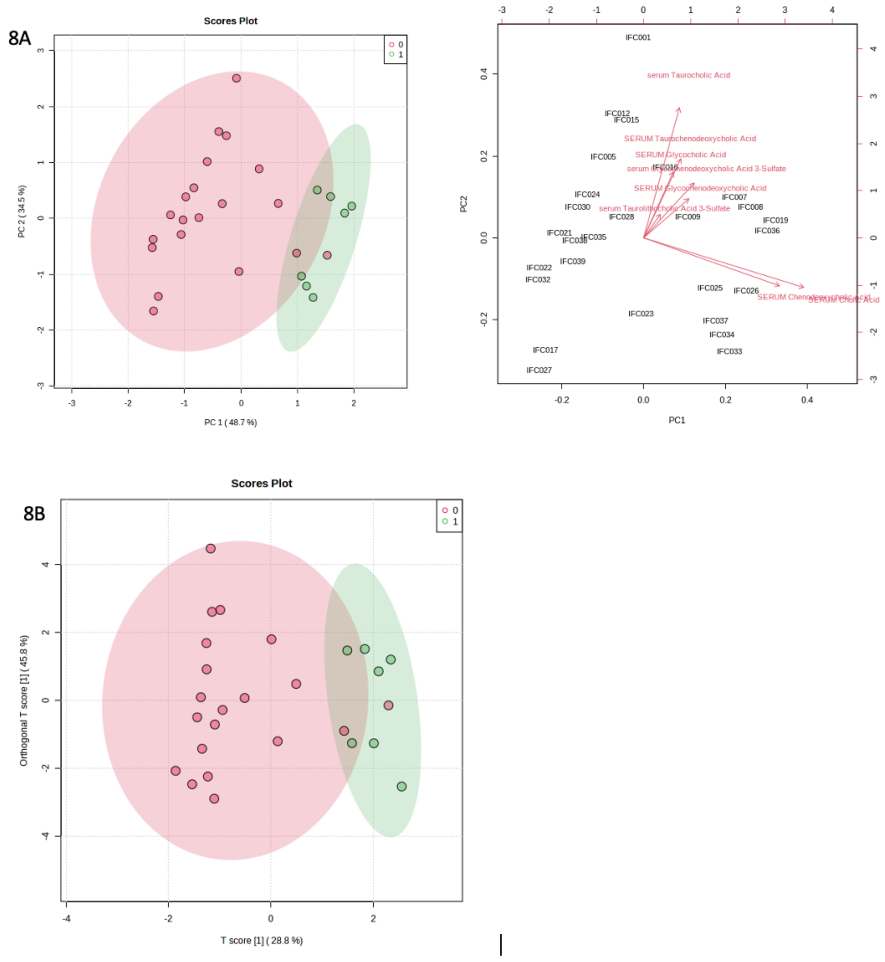


Table 18 Model validation for OPLS-DA for serum bile acids

R2X is fraction of variance of the x matrix; Q2 is whether the model has predictive ability.

	R^2X	Q^2	*p* value
Active vs remission	0.29	0.47	0.0008
Bowel length <150cm vs ≥150cm	0.19	-0.09	No good model
Enterostomy vs colon in continuity	0.20	0.43	0.25

6.6.4.2 Univariate analysis active disease vs remission

Serum from 19 patients in remission was compared to the serum from 7 patients with active disease. Univariate analysis showed enrichment with serum CA and serum CDCA in active disease compared to remission, (p=0.0004, Mann-Whitney test) (Table 20) (Figure 10). Serum CA and CDCA also correlated positively with CRP even when adjusted for covariates age, disease location, bowel length and BMI. The increase in serum PBAs with active disease seen are in keeping with previous studies in the published literature. There was one outlier patient with ultrashort bowel with 15cm of bowel who had very high cholic acid levels of 1637nM.

The conjugated derivatives: serum gCA, serum tCA and serum tCDCA and gCDCA were no different between remission and active disease; this was despite their unconjugated forms being 6-10-fold higher in active disease (Table 20).

Serum SBAs were <LLOQ in 70-100% of samples apart from tLCA-3-sulfate which was present in low concentrations. An almost complete absence of serum SBAs in patients with CD-IF is noteworthy.

Table 19 A)mean serum bile acid concentrations comparing remission with active disease, bowel length <150cm vs >150cm, and enterostomy vs colon in continuity.

CA, cholic acid; DCA, deoxycholic acid; gCA, glycocholic acid, gCDCA, glycochenodeoxycholic acid, tCA, taurocholic acid; tCDCA, taurochenodeoxycholic acid; LCA, lithocholic acid; pts, participants; gLCA, glycolithocholic acid; tLCA taurolithocholic acid; gDCA glycodeoxycholic acid; tDCA, taurolithocholic acid; UCDA, ursodeoxycholic acid; gUCDA, glycoursodeoxycholic acid; tUCDA, tauroursodeoxycholic acid; LLOQ, lower limit of quantification; ULOQ, upper limit of quantification.

A) Metabolite in serum	Remission	Active disease	P value	Bowel length <150cm	Bowel length >150cm	P value	Enterostomy	Colon	p

(mean)	N=20	N=7		N=20	N=7		N=20	In continuity N=7	
Serum primary bile acids (nM)									
CA	150	1555	**0.0004**	637	84.3	0.59	312	1090	**0.004**
CDCA	374	2366	**0.0007**	1096	174	0.43	466	2104	**0.002**
gCA	767	845	0.46	812	1229	0.18	798	763	0.91
gCDCA	961	1466	0.40	940.7	1361	0.21	885	1451	0.11
tCA	93	49	0.48	74	108	0.06	96	40	0.42
tCDCA	83.1	79	0.20	70	102	0.16	83	62	0.93
CA 3-sulphate	20/20 <LLOQ	7/7<LLOQ	Na	20/20 <LLOQ	7/7 <LLOQ	n/a	20/20 <LLOQ	7/7 <LLOQ	Na
tCA-3-Sulfate	19/20 <LLOQ	7/7 <LLOQ	Na	20/20 <LLOQ	7/7 <LLOQ	n/a	20/20 <LLOQ	7/7 <LLOQ	Na
gCA 3-Sulfate	15/20 <LLOQ	4/7 <LLOQ	Na	12/20 <LLOQ	7/7 <LLOQ	n/a	15/20 <LLOQ	4/7 <LLOQ	Na
CDCA 3-Sulfate	17/20 <LLOQ	1/7 <LLOQ	Na	12/20 <LLOQ	1/7 <LLOQ	n/a	16/20 <LLOQ	2/7 <LLOQ	Na
gCDCA -3-sulfate	82	133	**0.03**	114	64	0.93	90	110	0.24
tCDCA 3-Sulfate	18/20 <LLOQ	4/7 <LLOQ	Na	15/20 <LLOQ	4/7 <LLOQ	n/a	17/20 <LLOQ	5/7 <LLOQ	Na
Serum secondary bile acids (nM)									
LCA	20/20 <LLOQ	6/7 <LLOQ	Na	19/20 <LLOQ	7/7 <LLOQ	n/a	20/20 <LLOQ	6/7 <LLOQ	Na
DCA	19/20 <LLOQ	4/7 <LLOQ	Na	16/20 <LLOQ	7/7 <LLOQ	n/a	19/20 <LLOQ	5/7 <LLOQ	Na
gLCA	19/20 <LLOQ	7/7 <LLOQ	Na	19/20 <LLOQ	7/7 <LLOQ	n/a	20/20 <LLOQ	6/7 <LLOQ	Na
tLCA	20/20 <LLOQ	7/7 <LLOQ	Na	20/20 <LLOQ	7/7 <LLOQ	n/a	20/20 <LLOQ	7/7 <LLOQ	Na
gDCA	17/20 <LLOQ	4/7 <LLOQ	Na	18/20 <LLOQ	6/7 <LLOQ	n/a	20/20 <LLOQ	4/7 <LLOQ	Na
tDCA	19/20 <LLOQ	5/7 <LLOQ	Na	17/20 <LLOQ	7/7 <LLOQ	n/a	20/20 <LLOQ	4/7 <LLOQ	Na
UCDA	19/20 <LLOQ	1/7 <LLOQ	Na	13/20 <LLOQ	7/7 <LLOQ	n/a	18/20 <LLOQ	2/7 <LLOQ	Na
gUDCA	15/20 <LLOQ	117	Na	19/20 <LLOQ	5/7 <LLOQ	n/a	16/20 <LLOQ	1/7<LLOQ	Na

tUCDA	20/20 <LLOQ	6/7 <LLOQ	Na	19/20 <LLOQ	7/7 <LLOQ	n/a	20/20 <LLOQ	6/7 <LLOQ	Na
LCA 3-Sulfate	20/20 <LLOQ	6/7 <LLOQ	Na	19/20 <LLOQ	7/7 <LLOQ	n/a	20/20 <LLOQ	6/7 <LLOQ	Na
gLCA 3-Sulfate	17/20 <LLOQ	3/7 <LLOQ	Na	14/20 <LLOQ	7/7 <LLOQ	n/a	17/20 <LLOQ	3/7 <LLOQ	Na
tLCA 3-Sulfate	2.3	1.9	0.78	2.6	1.3	0.56	1.7	3.6	0.54
DCA 3-Sulfate	20/20 <LLOQ	6/7 <LLOQ	Na	19/20 <LLOQ	7/7 <LLOQ	n/a	20/20 <LLOQ	6/7 <LLOQ	Na
gDCA 3-Sulfate	19/20 <LLOQ	6/7 <LLOQ	Na	18/20 <LLOQ	7/7 <LLOQ	n/a	20/20 <LLOQ	5/7 <LLOQ	Na
tDCA 3 -sulfate	20/20 <LLOQ	7/7 <LLOQ	Na	20/20 <LLOQ	7/7 <LLOQ	n/a	20/20 <LLOQ	4/7 <LLOQ	Na
UCDA 3-Sulfate	20/20 <LLOQ	3/7 <LLOQ	Na	16/20 <LLOQ	7/7 <LLOQ	n/a	19/20 <LLOQ	4/7 <LLOQ	Na
gUCDA 3-Sulfate	14/20 <LLOQ	1/7 <LLOQ	Na	10/20 <LLOQ	6/7 <LLOQ	n/a	16/20 <LLOQ	3/7 <LLOQ	Na
tUCDA 3-Sulfate	20/20 <LLOQ	5/7 <LLOQ	Na	18/20 <LLOQ	6/7 <LLOQ	n/a	19/20 <LLOQ	6/7 <LLOQ	Na

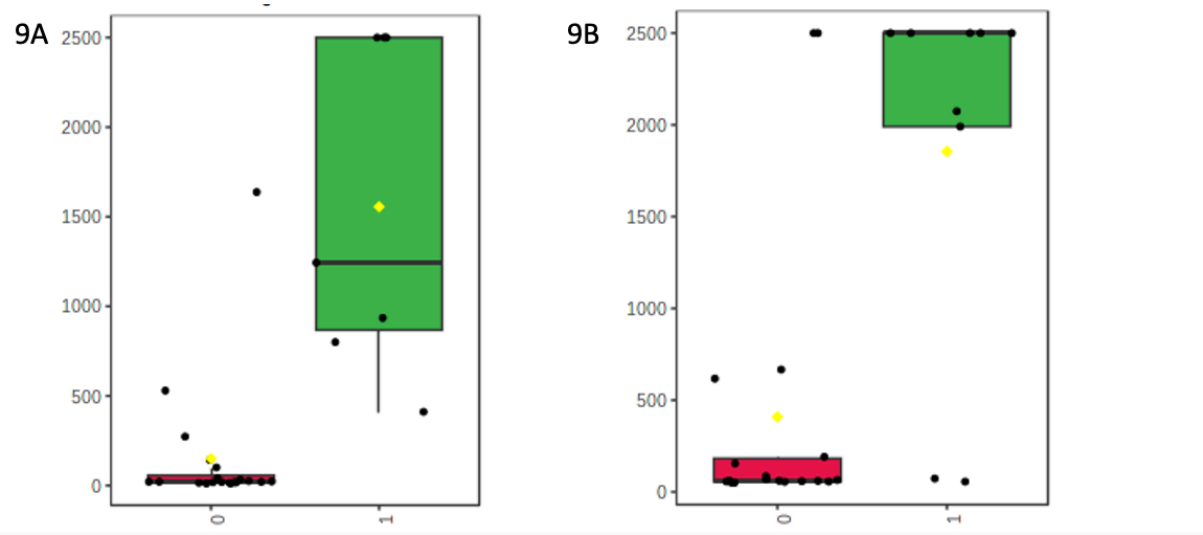
Table 20B) mean stool bile acid concentrations comparing remission with active disease, bowel length <150cm vs >150cm

B) Metabolite in stool	**Remission N=18**	**Active disease N=2**	**P value**	**<150cm N=14**	**>150cm N=6**	**P value**
Primary bile acids (faecal) (nM)						
CA	16/18>ULOQ	2/2 >ULOQ	Na	12/14 >ULOQ	6/6 >ULOQ	n/a
CDCA	12/18>ULOQ	2/2 >ULOQ	Na	10/14 >ULOQ	4/6 >ULOQ	n/a
gCA	15/18 >ULOQ	1/2>ULOQ	Na	10/14 >ULOQ	6/6 >ULOQ	n/a
gCDCA	15/18 >ULOQ	1/2> ULOQ	Na	10/14 >ULOQ	6/6 >ULOQ	n/a
tCA	10/18 >ULOQ	1/2>ULOQ	Na	6/14 >ULOQ	5/6 >ULOQ	n/a
tCDCA	9/18>ULOQ	1/2>ULOQ	Na	7/14 >ULOQ	5/6	n/a
CA 3-Sulfate	8/18<LLOQ	431	Na	6/14 <LLOQ	2/6 >ULOQ	n/a
CDCA 3-Sulfate	183	2/2>ULOQ	Na	225	360	0.80
gCA 3-Sulfate	89	500	0.87	167	188	**0.03**
gCDCA 3-Sulfate	389	502	0.43	266	715	0.52
tCDCA 3-sulfate	88	30	Na	79	90	0.04

tCA-3-Sulfate	5/18<LLOQ	7	Na	6/14 <LLOQ	1/6 <LLOQ	n/a
Secondary bile acids (faecal) (nM)						
DCA	46	75.4	0.51	50	51	0.55
gDCA	59	30	0.26	23	122	0.13
tDCA	10/18 < LLOQ	1/2 <LLOQ	n/a	6/14 <LLOQ	6/6 <LLOQ	n/a
LCA	5/18 <LLOQ	2/2 <LLOQ		9/14 <LLOQ	42	n/a
gLCA	21	13	0.40	15	34	0.27
tLCA	12	13	0.82	11	16	0.21
LCA- 3 sulphate	32	28	0.49	42	60	0.17
gLCA-3 sulphate	114	13	0.07	56	215	0.04
tLCA 3-sulfate	8/18 <LLOQ	1/2<LLOQ	n/a	5/14 <LLOQ	19.7	n/a
DCA-3 sulfate	16/18 <LLOQ	18	n/a	12/14 <LLOQ	4/6 <LLOQ	n/a
tDCA- 3 -sulfate	18/18 <LLOQ	2/2<LLOQ	n/a	14/14 <LLOQ	6/6 <LLOQ	n/a
gDCA- 3 -sulfate	9/18 <LLOQ	1/2 <LLOQ	n/a	9/14 <LLOQ	1/6 <LLOQ	n/a
UCDA	83	522	0.45	106	176	0.78
gUCDA	5/18 <LLOQ	1 /2 LLOQ	n/a	85	58	0.68
UCDA-3 sulfate	52	203	0.92	35	151	0.11
tUCDA-3 sulfate	18/18 <LLOQ	1/2 <LLOQ	n/a	13/14	6/6 <LLOQ	n/a

Figure 11 Serum A) cholic acid and B) chenodeoxycholic acid levels comparing remission(red) and active disease (green).

Centre of box- median, hinges – 25% and 75% quartiles and yellow dot mean.



6.6.5 Stool bile acids active disease vs remission

In stool, 69 bile acids were tested, however only 17 were present at detectable levels as most stool bile acids were in concentrations < LLOQ or >ULOQ for more than 20% of results (Table 20).

Whilst we are unable to make comparisons between active disease and remission because only 2/18 patients who submitted a stool sample had active disease, it is noteworthy that the stool primary bile acids (CDCA, CA, gCDCA, gCA, tCDCA, and tCA) were almost all >ULOQ. Conversely, stool secondary bile acids, DCA, gDCA, gLCA and tLCA were present in small concentrations and LCA and tDCA levels were almost all <LLOQ. This is in keeping with previous studies that in both IF and CD, there is increased faecal loss of PBAs and reduced conversion to SBAs. The excess of faecal PBAs could be related to a reduced surface area for reabsorption. The reduction in SBAs with an almost absence of LCA and DCA could be related to a lack of colonic bacteria due to absence or reduction of colonic surface area and due to reduction in diversity (Table 20).

A previous study (Boutte *et al.*, 2022) in patient with short bowel syndrome showed that conjugated derivatives: faecal gCA, tCA, tCDCA and gCDCA, were higher in patients with an enterostomy compared to those with a colon in continuity and the authors postulated that this may be because bacteria responsible for deconjugation reside in the colon.

6.6.6 Serum bile acids bowel length <150cm vs ≥150cm

There was no difference in serum bile acids when comparing CD-IF with small bowel length <150cm vs ≥150cm (Figure 11). The mean serum CA was 637nM amongst 20 patients with BL <150cm and 84nM amongst 7 patients with BL >150cm (p=0.59), but this did not reach significance on univariate analysis (Table 20). Similarly, the serum CDCA was higher in patients with BL <150cm compared to ≥150cm (1096nM vs 174nM), however, this also did not reach significance (p=0.43). Sample numbers were small.

When compared against continuous bowel lengths, there was an inverse relationship between serum CA and CDCA and bowel length (p=0.024 and p=0.016 respectively, Spearman's rho), indicating that the shorter the bowel, the higher the CA and CDCA.

Figure 12 Comparing serum bile acids detected by mass spectrometry in bowel length <150cm (red) vs >150cm (green). A) PCA B) OPLS-DA

6.6.7 Stool bile acids bowel length <150cm vs ≥150cm

We were unable to compare CD-IF with small bowel length <150cm vs ≥150cm owing to the finding that the stool SBAs were present in <LLOQ concentrations or very low concentrations in the majority of samples (Table 20). The stool PBAs were mainly >ULOQ. However, amongst those with detectable levels, the only significant finding was a higher glycocholic acid-3-sulfate in those with >150cm small bowel length (p=0.03).

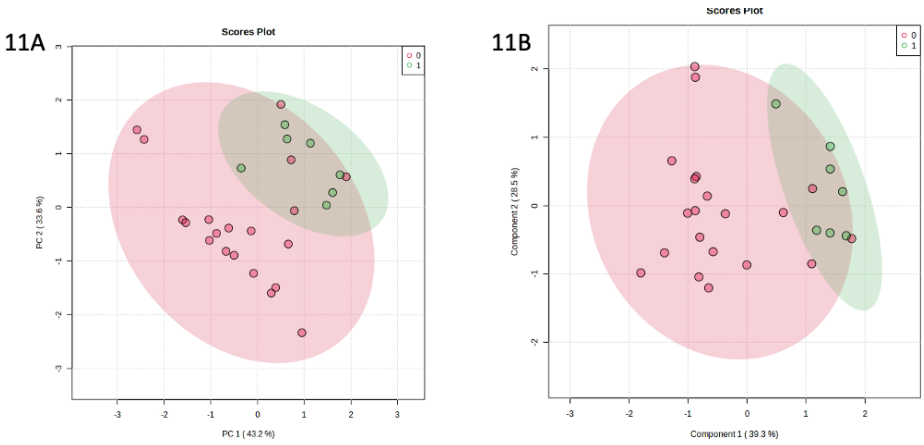
6.6.8 Serum bile acids enterostomy vs colon in continuity

Patient with a CIC (n=7) had significantly higher serum CA and CDCA, which were 3-5-fold higher than those with an enterostomy (n=20) which may be related to the CIC patients having more active disease (Figure 12).

Although we cannot perform statistics on serum SBAs because they were mainly <LLOQ, we note that they were more likely to be detectable, albeit at very low levels in the CIC group compared to the enterostomy group which is in keeping with our knowledge that conversion from PBAs to SBAs occurs in the colon.

Figure 13 Comparing serum bile acids detected by mass spectrometry in enterostomy (red) vs CIC (green)

A) PCA B) OPLS-DA



6.7 Results- metabonomic profiling of short chain fatty acids.

In serum, 2 SCFAs were analysed (serum lactate and 2-OH-butyrate). Acetate, propionate, isobutyrate and butyrate had missing data and therefore for not analysed.

In stoma effluent, 9 SCFAs were tested, and 6 were present in sufficient concentrations to be analysed including: lactate, acetate, propionate, hexanoate, 2-OH-butyrate and butyrate. 3 SCFAs were present mainly below the LLOQ and therefore not analysed: 2-Me-butyrate, isovalerate, valerate were not analysed.

In urine, 6 SCFAs were analysed (acetate, lactate, propionate, butyrate, 2OH butyrate, and isobutyrate). 2 Me Butyrate, isovalerate, valerate and hexanoate were not analysed as data was missing.

6.7.1 Paired stool and serum results for SCFAs

Only lactate and 2-OH butyrate were present in both stool and serum in sufficient concentrations to be compared. Stool and serum lactate did not correlate (n=14 paired samples, p=0.49), nor did stool and serum 2-OH- butyrate (p=0.32).

6.7.2 Comparing SCFAs between bowel length <150cm vs ≥150cm.

Stool lactate was higher in in those with small BL <150cm (Figure 13) by NMR, compared to those with ≥150cm. This was also seen on MS with stool lactate 4.1mM/mg in <150cm compared to 1.9nM/mg in >150cm, but this did not reach significance.

There was no difference in the serum, stool and urine SCFAs when comparing BL <150cm vs ≥150cm disease and remission (Table 21).

6.7.3 Comparing SCFAs between active disease vs remission.

There was no difference in serum, or stool, or urinary SCFAs when comparing active disease and remission (Table 21), however comparisons cannot be made as only 2/20 patients had active disease.

Table 20 A) Serum and B) stool and C) urinary short chain fatty acids in remission vs active disease and bowel length <150cm vs >150cm

A) Serum metabolite	**Remission (n=19)**	**Active disease (n=7)**	**Significance P value**	**<150cm (n=20)**	**>150cm (n=6)**	**Significance P value**
Lactate (mM)	44.9	36.6	0.13	40.6	48.2	0.79
Acetate (mM)	Missing data	Missing data		Missing data	Missing data	
Propionate (mM)	Missing data	Missing data		Missing data	Missing data	
2-OH-butyrate (µM)	8628.4	10353.8	0.80	9593	7714	0.39
Isobutyrate (µM)	Missing data	Missing data		Missing data	Missing data	
Butyrate (µM)	Missing data	Missing data		Missing data	Missing data	
B) Stool metabolite	**Remission (n=18)**	**Active disease (n=2)**	**Significance p value**	**<150cm (n=15)**	**>150cm (n=5)**	**Significance P value**
Lactate (mM/mg DW)	3.5	3.8	n/a	4.1	1.9	n/a
Acetate (mM/mg DW)	0.9	5.4	n/a	1.4	2.3	n/a
Propionate (mM/mg DW)	0.2	4.0	n/a	0.88	0.40	n/a
Butyrate (µM/mg DW)	31.1	19.7	n/a	28.2	31.1	n/a
2-OH-butyrate (µM/mg DW)	31.6	765.4	n/a	168	61	n/a
Hexanoate (µM/mg DW)	1.7	0.04	n/a	1.6	0.9	n/a
C)Urine metabolite	**Remission (n=21)**	**Active disease (n=6)**	**Significance p value**	**<150cm (n=22)**	**>150cm (n=5)**	**Significance P value**
Lactate (mM)	7.6	3.0	0.09	6.6	6.8	0.44

Acetate (mM)	Missing data	Missing data		Missing data	Missing data	
Propionate (mM)	Missing data	Missing data		Missing data	Missing data	
2-OH-butyrate (µM)	606	629	0.93	645	460	0.37
Isobutyrate (µM)	Missing data	Missing data		Missing data	Missing data	
Butyrate (µM)	Missing data	Missing data		Missing data	Missing data	

6.7.4 Correlations with body mass index

Urinary butyrate, 2-OH-butyrate and lactate correlate positively with higher BMI.

6.8 ^{1}H-NMR results

6.8.1 ^{1}H-NMR results of stoma effluent

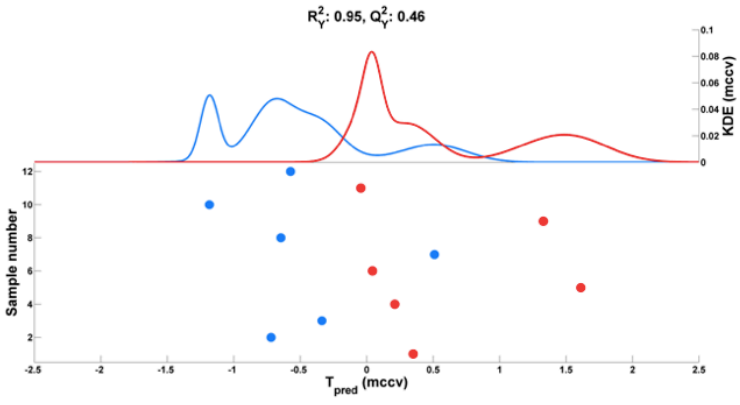
In stoma effluent NMR, 6 samples were excluded for the following reasons: presence of ethanol, obesity with BMI over 40, outlier bowel length 210cm, and one patient had glucose (possibly prediabetic).

There was no significant difference in the stoma effluent MCCV-PLS-DA full spectra when comparing: CRP <5 (n=9) vs CRP ≥5 (n=3), continuous CRP via PLS-Y model and continuous bowel length with PLS-Y model. Disease activity 1 year was not modelled as only 1 out of 12 included patients, who provided a stoma sample had active disease.

On NMR, stool oligosaccharides were higher in <150cm and stool valine was higher in ≥150cm (Figure 13).

Figure 14 1H-NMR stool spectra using MCCV-PLS-DA obtained from patients with CD-IF with different bowel lengths.

Patients with bowel length <150cm (blue, n=6) have higher stool lactate, phosphorycholine, glycophosphocholine and oligosaccharides. Patients with bowel length ≥150cm (red, n=6) had higher stool valine)



6.8.2 ^{1}H-NMR results of serum

There were 26 serum samples for NMR. There were no significant correlations on PLS-DA or PLS-Y model in the full NMR spectra for the following: bowel length <150cm vs ≥150cm, continuous bowel length, active vs remission 1-year, continuous CRP, continuous CRP in those with <150cm BL or enterostomy vs CIC. Subgroup analysis of enterostomy alone, and CIC alone also did not show any significant correlations.

On NMR, serum phosphocholine A and phosphocholine B, and HDL were higher in the enterostomy group (Figure 14) and GlycA and Glyc B were higher in the CIC group (Figure 15).
CRP correlated positively with *N*-acetylglycoprotein (GlycA) (q=0.0007) and GlycB (q=0.000074)) (Figure 15). CRP correlated negatively with serum phosphocholine A (q=0.0006) and phosphocholine B (q= 0.003).

The serum ApoB100:ApoA1 ratio was higher with CRP≥5 (Figure 16), q 0.018.

Figure 15 [1]H-NMR serum spectra using MCCV OPLS-DA. Patients with colon in continuity (red) had higher serum N-acetylglycoprotein A and B than patients with enterostomy (blue).

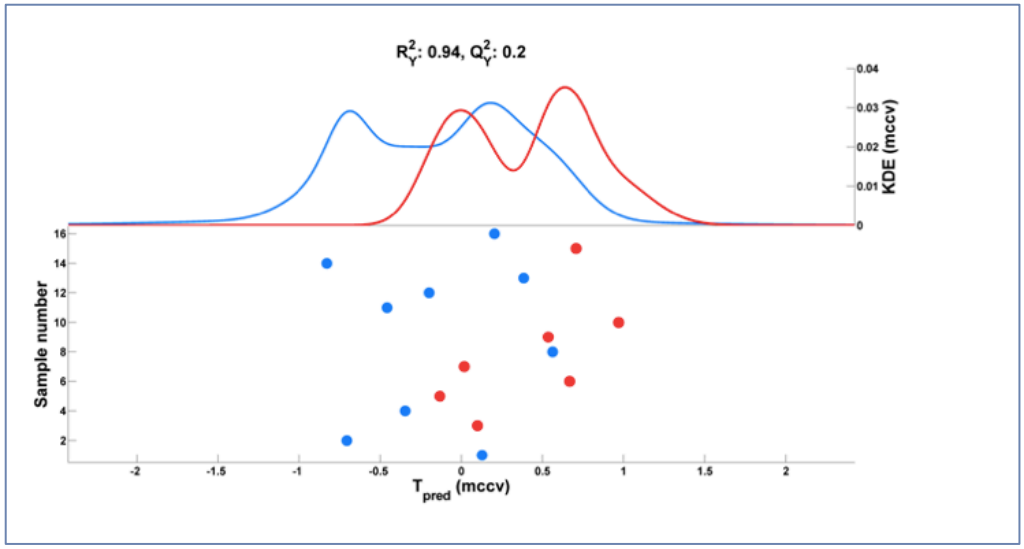


Figure 16 NMR spectra of serum correlation to CRP

CRP correlated positively with N aceylglycoprotein A and B (GlycA and Glyc B) and negatively with serum phosphocholine (SPC) A and B.

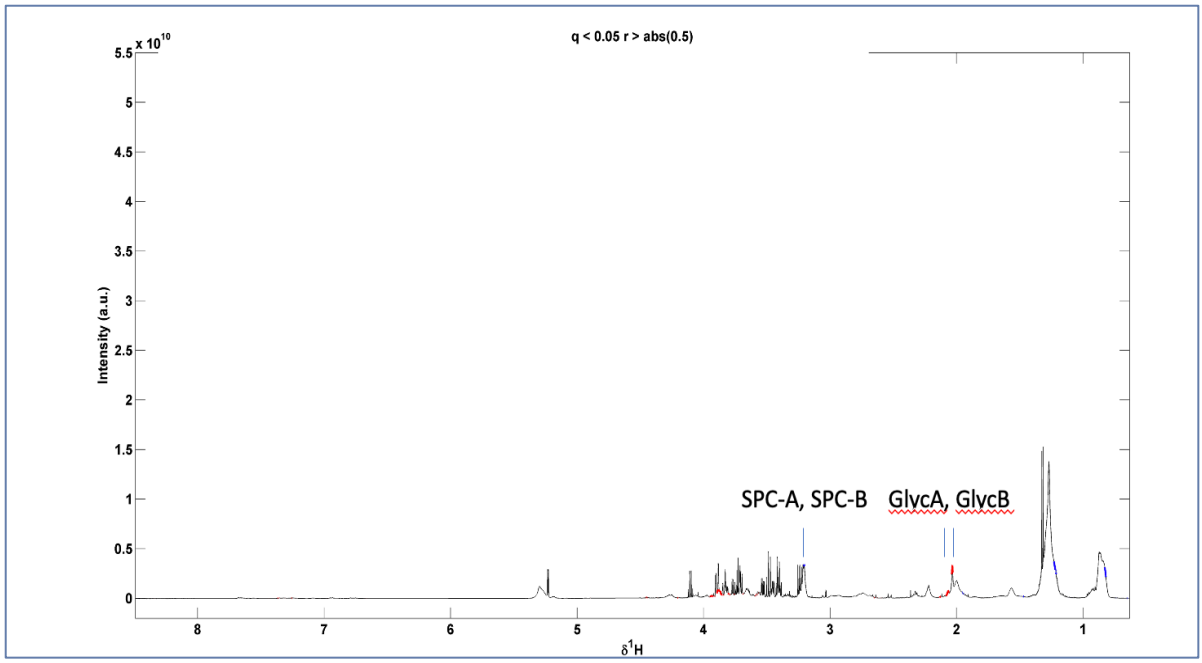

q < 0.05 r > abs(0.5)
x 10^10
Intensity (a.u.)
δ^1H
SPC-A, SPC-B
GlycA, GlycB

Figure 17 [1]H-NMR profiling of serum in CD-IF. a) table showing Apob100:ApoA1 ratio is increased in patients with CRP>5

A) Table showing Apob100:ApoA1 ratio is increased in patients with CRP>5. B) plot shown for all CD-IF patients and c) plot shown for bowel length <150cm. Wilcoxon rank sum test (Mann Whitney U test).

Model	R^2Y	Q^2Y	Feature	q	Association‡
MCCV-PLS-DA	0.65	0.31	Total Particle Number	0.054	↑
			LDL-Chol:HDL-Chol ratio	0.109	↑
			Apo-B100:Apo-A1 ratio	**0.018**	↑

‡Sign of association: **↑Upregulation for the group with CRP greater than or equal to 5**

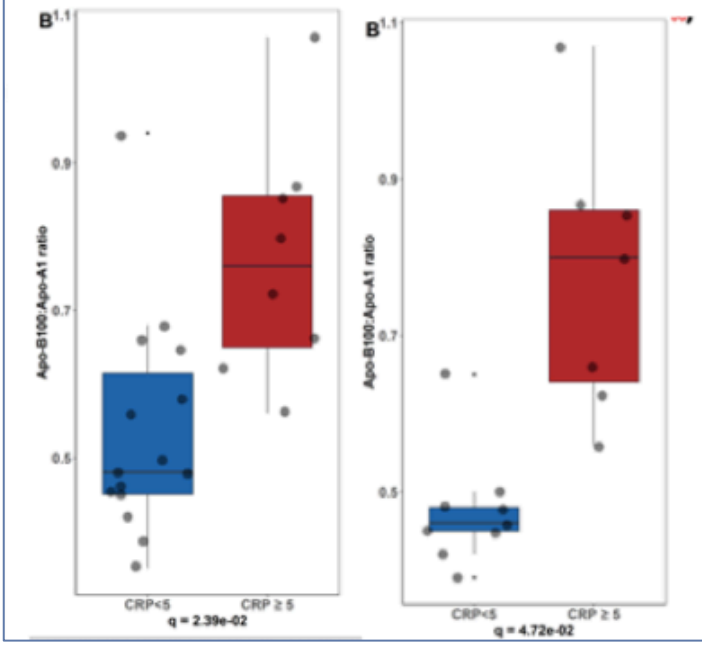
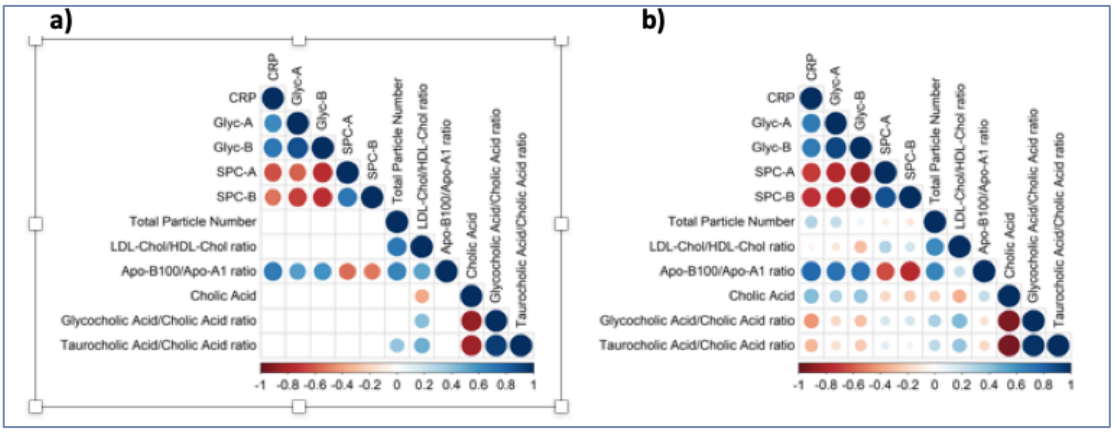


Figure 18 Rho Spearman correlation in CD-IF by 1H-NMR a) all length b) bowel length <150cm



6.8.3 ^{1}H-NMR results of urine

One patient was excluded with a BMI of 43 due to very high urinary sucrose levels.

In urine samples, no significant models or correlations were found when comparing bowel length <150cm vs ≥150cm, bowel length continuous, active vs remission, continuous CRP, CRP<5 vs CRP ≥5, enterostomy vs colon in continuity for <150cm bowel length, CRP in patients with bowel length than 150cm. There were no significant correlations between CRP continuous and full NMR spectra.

6.9 Discussion

6.9.1 Main clinical differences

Amongst 32 patients with CD-IF, the main clinical finding is that 92% of patients in the enterostomy group were in remission compared to only 29% in the CIC group. The CRP was also higher in the CIC group. Patients with CIC also had a shorter small bowel length (87.5cm vs 111.6cm), although this was not significant. 71% of patients with active disease had a surgical anastomosis with CIC compared to 8% of those in remission. Patients with an enterostomy were more likely to have L3 disease, which is expected as they may have had colectomy due to colonic involvement (Table 17).

6.9.2 Microbiota findings

Multiple studies have consistently shown that in health, the stool microbiome is dominated by *Firmicutes*, which makes up 60-80% and *Bacteroidetes* which makes up 20-40% (Ma *et al.*, 2022; Rinninella *et al.*, 2019; Ley, Peterson and Gordon, 2006). The MetaHIT study, which sequenced stool samples from 124 individuals, found that each individual carries around 160 different species (Qin *et al.*, 2010). There appears to be a spatial variation in microbiota composition as we move from the mouth, distally to the rectum in health; the number of bacteria increases and the bacterial profile changes too. The changes are thought to be driven by differences in the local luminal environment at different parts of the gut; the oxygen concentration decreases distally and there is a corresponding change with the presence of a greater number of obligate anaerobic bacteria. Several groups have attempted to measure the oxygen partial pressure of the gut lumen, and this has been challenging with varying results. There appears to be more oxygen in the stomach than in the sigmoid colon. One study showed that the intraluminal oxygen partial pressure in the mouse caecum is as low as 1mmHg (Albenberg *et al.*, 2014). Facultative anaerobes are organisms which can use oxygen but can grow with or without oxygen, whereas obligate anaerobes are unable to grow in oxygen. A study in 2009 (Hartman *et al.*, 2009), analysed the microbiota from patients with

ileostomies; they had an increase in facultative anaerobe, and when the ileostomies were reversed, there was an inversion with an increase in anaerobes and a decrease in facultative aerobes. This is in keeping with the theory that bacteria which grow in oxygen increase in patients with an ileostomy. The finding of increased *Lactobacillus* has been one of most reproducible and consistent findings among studies examining the microbiota in small bowel syndrome caused by mixed aetiologies (Davidovics *et al.*, 2016) (Huang *et al.*, 2017) (Joly *et al.*, 2010) (Mayeur *et al.*, 2013) (Boccia *et al.*, 2017) and although most of the patients in these studies had a jejunocolic anastomosis and not a stoma, the oxygen content is still likely to be higher given that they all had a short bowel. The oxygen theory was challenged by a group who compared the microbiota of ileostomy samples against samples obtained from the ileum of patients with a long oral catheter; there was a large proportion of *Clostridium Clusters XIV*, such as *Coprococcus* species, which are anaerobic. The authors remarked whether the differences seen in the ileostomy effluent were more likely due to the absence of colonic reflux, rather than a higher oxygen content (Zoetendal *et al.*, 2012)

In the oesophagus, duodenum and jejunum, *Streptococcus* predominates. In the colon *Firmicutes* and *Bacteroidetes* predominate; with a high abundance of *Bacteroides*, *Prevotella* and *Ruminococcus* (Jandhyala *et al.*, 2015). The normal microbiota composition of ileostomy effluent has been less well studied. Booijink and colleagues (Booijink *et al.*, 2010) reported the microbiota of 7 ileostomy effluent samples from patients without IF and compared them to 7 faecal samples from healthy individuals. The ileostomy effluent had a higher abundance of *Lactobacillus* and *Clostridiales*, *Veillonella*, and a lower abundance of *Ruminococcus* and *Bacteroides*. Zoetendal (Zoetendal *et al.*, 2012) examined the microbiota of 5 patients with ileostomies who had undergone colectomies, therefore still had their entire small bowel, apart from a few centimetres. He compared them to controls with an intact GI tract and sampled their ileostomy microbiota using an extended oral catheter. Healthy controls with an intact tract, had, at the ileal level, a predominance of *Clostridium clusters XIVa*, *Bacteroides* and *Clostridium cluster IV* and their jejunal samples was dominated by *Streptococcus* and *Clostridium clusters IX (Veillonella*), *Clostridium cluster XIVa* and *Proteobacteria*; the jejunal samples were more similar to the microbiome of ileostomy samples. Overall, the main organisms of the small bowel are *Streptococcus*, *Coliforms* and *Clostridium*. The creation of an enterostomy allows oxygen into an otherwise aerobic environment. Another study showed that in ileostomy effluent, there is a predominance of aerobic bacteria (Natori *et al.*, 1992), whereas in stool there are usually 100 times more anaerobic bacteria than there are aerobic bacteria (Marchix, Goddard and Helmrath, 2018).

Amongst our patients with CD-IF there was a shift in the microbiota, we observed a predominance of *Firmicutes* (relative abundance 76%) and *Proteobacteria* (18%) and a suppression of *Bacteroidetes* (2%) at the phylum level, whereas in health, Bacteroides is the second most predominant phylum. At a family level the predominant bacteria from the *Firmicutes* phylum were *Lactobacillus*, *Veillonella* and *Streptococcus*, whereas, in health the *Ruminococcus* and *Clostridium clusters XIVa* predominate. The expansion of *Lactobacillus* is seen consistently in SBS (Mayeur *et al.*, 2013; Cahova, Bratova and Wohl, 2017) and could be due to these aerotolerant anaerobes surviving in the increased oxygen content, and anaerobes such as Clostridium decrease. The second largest phylum was *Proteobacteria* (mainly accounted for by *Escherichia*). The suppression of *Bacteroidetes*, largely obligate anaerobes, could be explained by the increase in oxygen content. Another theory proposed is that as *Lactobacillus* ferments unabsorbed carbohydrates to convert it into energy, therefore may increase in order to increase energy harvest for enterocytes (Furtado *et al.*, 2013).

In our study, *Streptococcus parasanguinis* was positively associated with CRP. *S. parasanguinis* has been consistently associated with IBD in different studies. In one study of paediatric CD patients, one patient had very high *S. parasanguinis* counts (Kaakoush *et al.*, 2012). Another study (Baker, Jacob and Bowden, 2000) of patients with Crohn's arthropathy demonstrated antigens of *S. parasanguinis* in synovial fluid. A study of 34 patients with CD (Serrano-Gómez *et al.*, 2021) showed that *S. parasanguinis* was one of 11 species used to predict relapse in CD. A study in patients with UC (Hassan-Zahraee *et al.*, 2022), demonstrated a decrease in the faecal *S. parasanguinis* post-treatment. In one study. (Hall *et al.*, 2017), monthly stool samples from 20 IBD patients and 12 controls were collected; this showed a higher abundance of facultative anaerobes in IBD compared to controls. There were 15 species which were increased in this study including *S. parasanguinis.* To validate this cohort, they also analysed samples from the Lewis study (Lewis *et al.*, 2015); the organisms which were consistently increased in both cohorts included *S. parasanguinis, Ruminococcus gnavus* and *Streptococcus salivarius*. There was also a decrease in 6 anaerobes which was consistent across the longitudinal study and the Lewis cohort. The authors concluded that these differences could be explained by the tolerance of these organisms to oxidative stress.

Diallister, Erysipelotrichaceae and *Alistipes* correlated positively with X2-OH-Butyrate. Diallister belongs to the phylum *Firmicutes* and is known to produce Butyrate (Feng, Ao and Peng, 2018). A study by Taylor and colleagues (Taylor *et al.*, 2020) showed a correlation between *Diallister* and valerate which was higher in CD patients a faecal calprotectin <100µg/g. *Erysipelotrichceae* is a

Firmicute and has previously been shown to produce butyrate (Louis and Flint, 2017). *Alistipes* is a Bacteroidete and has previously be shown to produce Butyrate (Vital, Karch and Pieper, 2017)

6.9.3 Active disease vs remission

Patient with active disease had a similar mean small bowel length (106.5cm vs 115.7cm).

6.9.3.1 Bile acids.

1. **Serum CA and CDCA were higher in patients with active disease** (regardless of covariate 150cm and covariate of bowel length continuous). Increased serum PBAs in CD have also been shown in other studies (Gnewuch *et al.*, 2009; Scoville *et al.*, 2018b) (section 4.3). It has been suggested that active disease or bowel length shortening due to resection results in loss of FXR receptors which leads to reduced FGF19, and a loss of negative feedback inhibition of bile acid synthesis so there is an increase in hepatic synthesis causing serum levels of PBAs to rise. In support of this, a study (Boutte *et al.*, 2022) showed that short bowel patients had significantly lower levels of FGF19 compared to healthy controls. There was no difference in FGF19 between those with colon in continuity and those with a small bowel enterostomy. Stool conjugated PBAs were higher in patients with SBS without a colon as colonic bacteria deconjugate BAs. Patients with CD have also been shown to have reduced FGF19 (Wilson *et al.*, 2020). A study by Nolan (Nolan *et al.*, 2015) of 58 patients with CD showed that fasting serum FGF19 levels inversely correlated with the length of ileal resection. Also, amongst patients without ileal resection, FGF19 was lower in those with active disease compared to inactive disease (Jahnel *et al.*, 2014). FGF19 is thought to be anti-inflammatory; it reduces hepatic bile acid synthesis, and it reduces the local inflammatory response (Gadaleta *et al.*, 2020). FXR is more abundant in the terminal ileum, however mice studies have also shown some, albeit lower levels of expression in the duodenum and jejunum too. These findings support the proposed mechanism that patients with ileal resection, have reduced reabsorption of PBAs leading to higher faecal levels of PBAs. Several mechanistic links have been proposed by which bile acids can influence inflammatory activity through signalling molecules. One is that bile acids bind to pregnane X receptor (PXR) and activation of PXR increases tumour growth factor β**,** IL10, and reduces TNF- α expression. Another, possible mechanistic link, is that BAs bind to Takeda G-coupled receptor 5 (TGR5), which is present in the small intestine and colon, with the highest affinity being for LCA, then DCA, CDCA, UCDA and CA. TGR5 is thought to reduce intestinal inflammation. Mice treated with BAs had reduced expression of pro-inflammatory cytokines, including TNF-α and increased expression of anti-inflammatory genes (Bromke and Krzystek-Korpacka, 2021).

2. **Faecal PBAs in CD-IF were very high**, almost all were >ULOQ. This is a consistent finding in the published literature (Franzosa *et al.*, 2019; Lloyd-Price *et al.*, 2019) (Kolho *et al.*, 2017) and a plausible explanation is that a reduced reabsorption leads to higher faecal loss and also there is reduced conversion of PBAs to SBAs.
3. **There was almost an absence of stool SBA**, with most of them being undetectable. This is a consistent finding in the literature in patients with CD. The leading theory is that there is reduced conversion of PBAs to SBAs due to a lack of colonic bacteria.
4. There was an almost **complete absence of sulphated bile acids in both stool and serum (Table 21).** We are unable to perform analysis as almost all were present in concentrations <LLOQ and only 2 patients in the faecal sample group had active disease. Bile acids are sulphated to increase their solubility, reduce their absorption, and reduce their toxicity. A study (Jahnel *et al.*, 2014) demonstrated that patients with active CD had lower expression of SULT2A1 expression, which is a sulphating enzyme, compared to patients in remission and controls, therefore sulphated bile acids would be expected to be lower in active disease. Other studies (van der Lugt *et al.*, 2022) have shown that patients with IBD have a reduced desulphation capacity and have higher sulphated bile acids. Desulphation is thought to occur in the colon therefore sulphated bile acids may be higher in patients who lack a colon. A study by Ding and colleagues (Ding *et al.*, 2020b) showed that non responders to anti-TNF therapy had higher levels of sulphate- conjugated PBAs. Therefore, the role of sulphation and desulphation in CD is not clear and in future studies, sulphated bile acids deserve attention.

6.9.3.2 SCFAs

Acetate is produced by microbes catabolising dietary carbohydrates and also by the synthesis of acetate from acetogenic bacteria. It has anti-inflammatory properties. Normally, acetate is the most abundant SCFA in the colon (Louis *et al.*, 2007), however, we noted an almost complete absence of acetate which is noteworthy.

6.9.3.3 Lipids

Serum phosphocholine lipids correlated negatively with CRP (Figure 15). Phosphocholine (PC) is a component of the cell membrane and is involved in cell signalling. Phosphocholine lipids play key roles in the modulation of inflammation and immunity. A study by Ding (Ding *et al.*, 2020b) of 76 patients with Crohn's disease treated with anti-TNF therapy were categorised as responders, non-

responders or partial- responders. Phosphocholines, ceramides, sphingomyelins and triglycerides and primary bile acids were higher in serum and faeces of the non-responders.

6.9.3.4 Proteins

Glycoprotein A and glycoprotein B correlated positively with CRP on NMR (Figure 15).
This was consistently seen when all bowel lengths were analysed. GlycA is the name given to the H^1-NMR signals that originate from a group of certain glycosylated acute phase proteins such as α1-acid glycoprotein, haptoglobin, α1-antitrypsin and α1-antichymotrypsin(Connelly *et al.*, 2017) . GlycA has consistently been reported as a promising new biomarker of active inflammation across many studies, including cross sectional studies (Akinkuolie *et al.*, 2014; Otvos *et al.*, 2015b) and in chronic inflammatory conditions such as rheumatoid arthritis, psoriasis and lupus and also in cardiovascular disease (Otvos *et al.*, 2015a; Akinkuolie *et al.*, 2014). *N*-acetylated glycoproteins were upregulated in CD compared to controls (Martin *et al.*, 2017; Williams *et al.*, 2012a). Multiple authors also report that GlycA is better at predicting inflammation than CRP (Fuertes-Martín *et al.*, 2020). GlycA has been found to be a sensitive marker of SARS-CoV-2 positive patients (Lodge *et al.*, 2021). A study in 2019 with 37 CD, 21 UC and 10 controls showed that GlycA levels were significantly higher in patients with active disease compared to those in remission. GlycA also correlated with CRP and faecal calprotectin levels (Dierckx *et al.*, 2019). Further larger studies specifically in IBD are required. GlycB is the name given to the signal arising from a group of glycosylated proteins and has also been shown to be associated with CRP (Lorenzo *et al.*, 2017) however there is less data about GlycB.

ApoB100:ApoA1 ratio is increased in patients with CRP≥5 (Figure 16). There was a significantly increased Apo-B100: Apo-A1 ratio in patients with CRP ≥ 5 mg/dL, this change was confirmed by univariate analysis. The ApoB100:apoA1 ratio is a strong predictor of cardiovascular disease. ApoA1 is the most abundant component of high-density lipoprotein (HDL) and is anti-inflammatory; whereas ApoB is a component of LDL and is pro-inflammatory. In one study, patients with ileal CD had downregulation of the APOA1 gene which blocks the activation of monocytes leading to the inhibition of IL-1β and TNF-α production (LaFlamme, 2014). Another study showed that CD patients who had a response or a primary non-response to infliximab had an upregulation of ApoA1 compared to pre-treatment levels (Gazouli *et al.*, 2022).

Serum phosphocholine A and B are potential anti-inflammatory markers; they correlated negatively with CRP and were higher in patients with enterostomy.

6.9.4 Correlations with bowel length

There was no difference in bile acids when comparing those with BL <150cm vs ≥150cm, but univariate analysis, however, when compared against continuous bowel lengths, there was an inverse relationship between serum CA and CDCA and bowel length (p=0.024 and p=0.016 respectively, Pearson's coefficient). However, the numbers of patients with BL ≥150cm are small and the data is heterogenous with 28% having active disease in the <150cm group with no patients with active disease in the ≥150cm group and there were different proportions of patients with an enterostomy.

6.9.5 Correlation with anatomy

Patients with a CIC were more likely to have active disease in the last year compared to those with an enterostomy (71% vs 8%%) and had a higher mean CRP (8.4 vs 3.3 mg/dl). Serum CA, CDCA were higher in CIC which is likely to be attributed to there being more active disease in the CIC group. Notably, faecal SBAs are near absent in CD-IF but were more likely to be present in low concentration in the CIC group as colonic bacteria convert PBAs to SBAs.

6.10 Limitations of the study

Limitations of the study include the small sample size and lack of patients with active disease due to the finding that patient's disease activity often remits once intestinal failure develops. The small sample size made it impossible to compare the microbiota between those with active disease and those in remission. There are confounders, such as geographical location, diet, ethnicity and number of nights on PN which can introduce bias. There was also no longitudinal study with collection at a later timepoint. We also did not have stool samples from patients with CD-IF with no stoma as the time to centrifugation would have been too long as most of these patients live a few hours away. There was a lack of dietary data in order to detect confounders or associations. Only 13 patients were able to provide both stoma effluent and serum.

Whilst omics studies are an invaluable tool, the samples are sensitive to degradation depending on external conditions including temperature, freeze-thaw cycles and time to centrifugation which can introduce potential error. The wealth of studies with thousands of molecules being detected with high throughput methods, inconsistent results are seen, which is not surprising. This highlights the need to control confounders and optimise sample handling conditions. Each biofluid may contain thousands of compounds and their analysis requires complex pipelines; some compounds have not

been identified in the database and are therefore 'unknown' and introduces a detection bias(Richelle, Joshi and Lewis, 2019).

7 Conclusion

In our large retrospective study of 139 CD-IF patients, we describe a group of patients who are mostly in remission; 105 patients were in remission and 34 had active disease. We found that the presence of a surgical anastomosis was associated with active disease (OR 4.3, p=<0.001). When compared to 100 patients with CD (without IF) we found that being female, having ileocolonic disease, penetrating disease, or never having had an adequate trial of biologics were risk factors for developing IF.

The main finding of our multiomics study of 32 patients with CD-IF is that patients with a colon in continuity (CIC) are more likely to have active disease and a higher CRP than patients with an enterostomy. In CD-IF, the microbiota shifts to one which is predominantly composed of *Firmicutes* and *Proteobacteria*, which are mainly facultative anaerobes and aerotolerant anaerobes; with a suppression of the *Bacteroidetes* phylum, which normally predominates in health, but is composed largely of obligate anaerobes. These changes may be due to probable higher oxygen concentration of the lumen which we expect would occur after short bowel and enterostomy formation as 78% of our cohort had an enterostomy (jejunostomy or ileostomy). *Veillonella*ceae and *Lactobacillaceae* were the most predominant organisms which are facultative and aerotolerance anaerobes.

Our study focussed on bile acids as they can provide insight into mechanistic pathways and are known to be linked to inflammation. The main finding to highlight is that serum PBAs (CA and CDCA) were higher in active disease and were inversely proportional to bowel length. This finding has been seen consistently in other studies in both CD and IF. It is proposed that loss of FXR receptors in CD-IF due to ileal resection or inflammation, results in reduced FGF-19 and therefore a reduced negative feedback inhibition on hepatic synthesis of PBAs. Patients with CD-IF have a near absence of serum and stool secondary bile acids (SBAs) which is an expected finding as 78% of patients in this cohort did not have a colon which is where the bacteria which convert PBAs to SBAs. 7 patients who did have a CIC, had only <50% or >50% of colon remaining.

Our finding of the association between *Streptococcus parasanguinis* and CRP have been shown before. One theory is that *S. parasanguinis* can thrive after the increase in the oxygen concentration in the bowel after surgery or the increase in reactive oxygen species due to inflammation.

In addition to serum CA and CDCA described above, we have identified other potential pro-inflammatory biomarkers, which were associated with CRP or active disease including: serum GlycA and serum GlycB, serum Apo-B100: Apo-A1 ratio. The potential anti-inflammatory biomarkers identified include serum phosphocholine A and B.

Most of the findings support those of previous studies and were expected in keeping with what we already understand about the pathophysiology of the gut microbiome and bile acids. It remains unclear what the mechanistic explanation is, for the high remission rate is that we observe in severe CD patients, once IF sets in. Our cohort only had a total of 7 patients with active disease.
Potential candidates for remission inducers include changes in diet, microbiota shifts due to increased luminal oxygen or bile, the lack of a surgical anastomosis. These potential candidates deserve further attention in future studies.

8 Future work

I have identified areas I would like to focus on in future work. In the first retrospective study, I compared 100 patients with CD to 139 patients with CDIF. I would like to increase the number of control patients with CD to improve the power of the study. I would also like to focus on those patients with upper GI disease and see if they were more likely to progress to higher Montreal classifications. I would like to have a second blinded assessor rate the aetiology of intestinal failure as there is an inconsistency in the way the few published studies have assigned aetiology; whether it is felt to be due to active disease, or a surgical complication. Part of the discrepancy arises because, in many patients, it is difficult to say whether the patient would still have gone on to develop if, had the surgical complication not occurred. A consensus in the categorisation of the cause of IF may be helpful to understand why patients are developing IF.
Finally, it would be useful to further examine the finding that patients who undergo continuity surgery are more likely to develop active disease. I would like to compare post

operative medication use between groups and whether these were prophylactic or reactive to the development of active disease.

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9 Supplementary materials

Supplementary material 1 Patient information sheet

25/09/20157

Division of Medicine

PARTICIPANT INFORMATION SHEET

Study: Characterising the microbial and metabolic profile in patients with Inflammatory Bowel Disease (IBD)

Chief Investigator: Dr Horace Williams, Professor Ailsa Hart

BLOOD, URINE, STOOL SAMPLES

Dear Sir/Madam,

You are being invited to take part in a research study; please take time to read the following information carefully. Ask us if there is anything that is not clear. Participation is voluntary and you will be given a copy of this information sheet and your consent form. Thank you for reading on.

What is the purpose of this study?

We are looking into the causes of inflammatory bowel disease (IBD) which includes Ulcerative Colitis and Crohn's disease. Previous research has suggested that the bacteria that live our bowel, and their metabolites play an important role in this disease. This research aims to study this in more detail, and to see whether the bacteria that live in the bowel may also affect who develops IBD and whether microbial and metabolic profile might be able predict the type of IBD, its course and response to treatment.

Why have I been chosen?

You have been selected as you have either Crohn's disease or Ulcerative Colitis.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part, you will be asked to sign a consent form. You can withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What do I have to do?

If you agree to participate, this will involve us collecting a blood sample (approximately 15ml, usually at the same time as routine blood tests), a urine, and a stool sample plus a 5–10-minute chat to a doctor to go through a questionnaire.

We also plan to take urine, stool and bloods samples again after any new medications are started or after any changes to your medications or at clinic appointments. This will also be entirely your choice. We will ask you to complete a short questionnaire each time we collect samples, which will take

around 5 minutes. This will allow us to verify your diet and medications and understand the data we analyse.

If you are due have a colonoscopy and if biopsies are being taken as part of your standard care, we may ask you if we can take a couple of extra biopsies. Taking extra biopsies does not increase your chance adverse events over standard care.

Data analysis will involve accessing your patient records and notes.

There are no lifestyle or dietary restrictions, and you should continue on your current medication as directed. You should not however be involved in any other drug studies at the same time. Involvement in this research will in no way affect your treatment.

What if something goes wrong?

We do not anticipate any adverse effect as we are simply collecting samples which is not associated with any risk.

Imperial College holds Public Liability ("negligent harm") and Clinical Trial ("non-negligent harm") insurance policies which apply to this trial. If you can demonstrate that you experienced harm or injury as a result of your participation in this trial, you will be eligible to claim compensation without having to prove that Imperial College is at fault. If the injury resulted from any procedure which is not part of the trial, Imperial College will not be required to compensate you in this way. Your legal rights to claim compensation for injury where you can prove negligence are not affected.

Will taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised from it.

Procedures for handling, processing, storage and destruction of their data are compliant with the Data Protection Act 1998.

What will happen to the results of the research study?

The results of this research will have no implications for individuals, and the samples will be analysed anonymously. The research will give us a greater understanding of bowel disease, and why different people get different problems, and it may in future lead to the development of new treatments. Samples will be stored and may be used in future research.

We aim to publish the results of the study(s) in 2-3 years' time. You will not be identified in any report or publication but if you would like to obtain details/a copy of the published results, please contact the number detailed below for the GI unit (02078861072).

Who is organising and funding the research?

The sponsor for the research is Imperial College and the doctors are not being paid for including you in this study.

Who has reviewed this study?

This study was given a favourable ethical opinion for conduct in the NHS by REC.

Contact for further information.

If you have any questions, please contact:

Dr Shiva Radhakrishnan, GI Unit, St Mary's Hospital, Praed St, London W2 1NY. Tel: 0207 886 1072 or

Dr Sonia Bouri, St Mark's Hospital, Watford Road, Harrow, HA1 3UJ.Tel: 0208 869 5808

Thank you for reading this information sheet.

A copy of the written information sheet and signed informed consent form will be given to the participant to keep.

Supplementary material 2 -metabonomics questionnaire

Imperial College London

IBD URINARY METABONOMICS: QUESTIONS

Name:…… **Hospital Number:**…….

Date:…… **Urine type:**…… **BMI:**.....

1. CURRENT MEDICATIONS (Meds in past 7 days, name & dose)

ANY OTHER MEDICAL PROBLEMS:

Herbal remedies?

2. DIET (past 24 hours & usually)**:**

Vegetarian?		Cherries?	Carbonated drinks?
Meat (red/white/amount)?		Cheese?	Yoghurt?
Fish (type/amount)?		Grapefruit?	Berries?
		Liquorice?	
Coffee?	Milk?	Walnuts?	
Tea?	Herbal?	Vanilla?	

2. Last food (hours previously & nature)**:**

3. ALCOHOL (units/week & past 24 hours)**:**

4. SMOKING (cigs/day & duration; ever smoked?)**:**

5. EXERCISE in past 24 hours (none/light/medium/hard)**:**

6. MENSTRUAL CYCLE (LMP date & cycle length)**:**

7. DISEASE ACTIVITY:

CD: i. General well being (0=well, 1=slightly under par, 2=poor, 3=very poor, 4=terrible)

ii. Abdominal pain (0=none, 1=mild, 2=moderate, 3=severe)

iii. Number of liquid stools per day

iv. Abdominal mass (0=none, 1=dubious, 2=definite, 3=definite & tender)

v. Complications (arthralgia/-itis, uveitis/iritis, EN, PG, aphthous ulcers, anal fissure, new fistula, fever, abscess)

UC: i. General well being (0=well, 1=slightly under par, 2=poor, 3=very poor, 4=terrible)

ii. Bowel frequency day (1-3, 4-6, 7-9, >9)

iii. Bowel frequency night (0, 1-3, 4-6)

iv. Urgency (none, hurry, immediately, incontinence)

v. Blood in stool (none, trace, occasionally frank, usually frank)

vi. Complications (arthralgia/-itis, uveitis/iritis, EN, PG, aphthous ulcers, anal fissure, new fistula, fever, abscess)