Imperial College London

METABONOMIC PROFILING IN INFLAMMATORY BOWEL DISEASE: APPLICATION TO REAL-LIFE POPULATION OF PATIENTS.

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For the degree of Doctor of Medicine

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2021

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Statement of originality

I certify that this thesis, and the research to which it refers, are the product of my own work, and that any ideas or quotations from the work of other people, published or otherwise, are fully acknowledged in accordance with the standard referencing practices of the discipline.

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This thesis is dedicated to my wife Hayley, and children Jack and Daisy.

With love.

Abstract

Previous studies have demonstrated the importance of the microbiome in the pathogenesis of inflammatory bowel disease (IBD), with a dysbiosis being a common feature. This change in the microcbiome has metabolic consequences, and metabonomic profiling can be used to detect these metabolic signals along with changes in the patient's own metabolic profile. Metabonomics have been applied clinically in oncology to augment diagnosis, treatment, and prognostication, and the aim is for it to have a similar role in IBD. Most studies in IBD have been performed on stool, and have observed a number of discriminatory metabolites, but more recently urinary metabonomics has been studied, as this is a much more convenient biofluid to use both in terms of sample collection and preparation. Here too, discriminatory metabolites have been observed, several of which are produced by intestinal microbial or host-microbial metabolism. These changes in the IBD metabolome give an insight into the complex pathophysiology of this disease, and have significant potential impact in both diagnosis and stratification of patients into different disease phenotypes and in the assessment of treatment outcome.

However, up until now IBD metabonomic research has been restricted to homogeneous clinical cohorts where subjects with comorbidities have been excluded, and for the technique to be clinically useful it has to provide consistent results in real-life cohorts, including factors that may influence the metabolome such as obesity and the effects of bowel cleansing pre-colonoscopy, both of which apply to a real-life IBD clinical population. This project assessed these factors on the IBD metabolome, examining principally urine due to its ease of use.

Multivariate analysis showed that changes seen in the IBD metabolome are present with the inclusion of subjects with comorbidities in IBD and Crohn's disease cohorts relative to controls. Targeted analysis showed differences in hippurate and 4-cresol sulfate between IBD

cohorts relative to controls, likely associated with reduced abundance of *Clostridia* species seen on microbiomic analysis. No changes could be seen in ulcerative colitis (UC) cohorts, but UC participant numbers were relatively low following removal of patients taking 5-aminosalicylates that influenced the NMR spectrum.

Obesity may have an effect on the urinary IBD metabolome, as although there was no clustering on unsupervised multivariate analysis, a reduction in hippurate excretion was not seen in the obese IBD relative to control cohorts, and hippurate has been the most consistently reported discriminatory metabolite associated with IBD.

Longitudinal analysis of the urinary and faecal metabolome showed resistance at day 3 and week 6 following bowel cleansing; a temporary reduction of alpha diversity was observed on microbiomic analysis.

Overall, this project has shown that whilst many elements of the IBD related urinary metabolome are preserved when patients with comorbidities are included into analysis, obesity may be a significant confounder. The studies of the urinary and faecal metabolome after bowel cleansing show that the metabolome appears resistant to significant changes at day 3 following bowel cleansing, which may be helpful in analysing future metabolic and microbiomic studies after colonoscopy, and help to associate changes identified from sampling during those procedures.

Acknowledgements

Firstly, I would like to thank my primary research supervisor Professor Tim Orchard, who has very much been my career mentor too. He has guided me through my research period, and has provided excellent support over the years, as well as helping me develop my clinical subspecialty and patiently teaching me colonoscopy! Next, I would like to thank Dr Horace Williams, firstly for getting me started in the early days of research by teaching me the finer points of keeping a well-maintained Excel metadata spreadsheet. His support along the way, and insights into resolving shop-floor research problems have been invaluable. Dr Lucy Hicks, a senior research fellow colleague, has been a great friend and has given me enormous support over the years.

I would like to thank Kate Gallagher, whose support from the metabonomic analysis perspective has been paramount for this project. I am grateful for support from Dr Jonathon Swann, Dr Beatriz Jimenez, and Dr Jia Li, who have also been very helpful with the complex metabonomic analysis aspect of this work. Pav Rai, a fellow researcher in the early days, helped me greatly in getting started in the laboratory for which I am very thankful.

I am also indebted to Professor Mark Thursz for allowing me research space and use of the microbiology laboratory in the St. Mary's Hospital Academic Liver Unit. Help in microbiomic analysis from Dr Julie MacDonald, Dr Benjamin Mullish, and Dr James Alexander is also much appreciated. I developed some wonderful friendships during my research time in the Liver Unit, including Dr Nowlan Selvapatt, Dr Ameet Dhar, and Dr Harry Antoniades. Harry sadly passed away before seeing me submit this thesis, and I am sure he would be proud that I have done so – you are sorely missed.

The unwavering support from Hayley, my wife, has made completion of this thesis possible. My two children, Jack and Daisy, have often had to endure "daddy working" at home, and to them I am so thankful.

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List of abbreviations

^{1}H	Proton
5-ASA	5-aminosalicylate
6-MP	6-mercaptopurine
6-TGN	6-thioguanine nucleotides
16S rRNA	Small-subunit (16S) ribosomal RNA gene
Δ	Chemical shift
AAD	Antibiotic associated diarrhoea
ATG16L1	Autophagy-related 16 like 1 gene
AUC	Area under the curve
B_0	Nuclear spin in the presence of an external magnetic field
B_1	Radiofrequency pulses used in NMR
CARD15	Caspase recruitment domain-containing protein 15
C	Non-IBD subjects including subjects with comorbidities
Cau	Caucasian
Cc	Non-IBD subjects with comorbidities
CD	Crohn’s disease
CDc	Subjects with Crohn’s disease and a comorbidity
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
D_2O	Deuterium oxide
DESI	Desorption electrospray ionisation
DMG	Dimethylglycine
DNA	Deoxyribonucleic acid
DSS	Dextran sulfate sodium
FCP	Faecal calprtotectin
FDR	False Discovery Rate
FID	Free induction decay
FXR	Nuclear farnesoid-activated X receptor
GC-MS	Gas chromatography – mass spectroscopy
GI	Gastrointestinal
GWAS	Genome-wide association studies
H	Healthy control
HBI	Harvey Bradshaw Index
IBD	Inflammatory bowel disease
IBDU	IBD unclassified
IBDc	Subjects with IBD and a comorbidity
IBS	Irritable bowel syndrome
ICR-FT/MS	Ion cyclotron resonance Fourier transform mass spectrometry
I	Nuclear spin
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
IPAA	Ileal pouch-anal anastomosis
JAK	Janus kinase
LC-MS	Liquid chromatography – mass spectroscopy
LDA	Linear discriminant analysis
m/z	Mass : charge ratio

MDAS	Mayo disease activity score
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor-kappaB
NGS	Next generation sequencing
NMR	Nuclear magnetic resonance
NNT	Number needed to treat
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
OPLS-DA	Orthogonal partial least squares-discriminant analyses
Oth	Other ethnic background (not Caucasian or South Asian)
PAG	Phenyacetylglutamine
PBS	Phosphate buffer saline
PEG	Polyethylene glycol
ppm	Parts per million
Q-TOF-MS	Quadrupole time-of-flight mass spectrometry
REIMS	Rapid evaporative ionisation mass spectrometry
RF	Radiofrequency
ROC	Receiver operating characteristic
PLS-DA	Partial least squares-discriminative analysis
SA	South Asian
SCCAI	Simple clinical colitis activity index
SCFAs	Short-chain fatty acids
SMAD7	SMAD protein family type 7
STAT3	Signal transducer and activator of transcription 3
T1	Time taken for spin-lattice relaxation
T2	Time taken for the spin-spin relaxation
TCA	Trichloroacetic acid
TGF	Transforming growth factor-β
TLR	Toll-like receptors
TNF	Tumour necrosis factor
Th1	Type 1 T helper cell
TMA	Trimethylamine
TMAO	Trimethylamine-N-oxide
TSP	3-trimethylsilyl-1-1-(2,2,3,3,-2H4) propionate
UC	Ulcerative colitis
UCc	Subjects with ulcerative colitis and a comorbidity
UK	United Kingdom

Chapter 1: Introduction

1.1 Inflammatory bowel disease

1.1.1 Epidemiology and diagnosis

Inflammatory bowel disease (IBD), encompassing ulcerative colitis (UC) and Crohn's disease (CD), is a chronic immune-mediated disease that causes inflammation of the gastro-intestinal (GI) tract (1). UC is a colonic disease characterised by continuous mucosal inflammation starting in the rectum, and with varying extension through the colon. It follows a relapsing and remitting course, and histologically there is usually the absence of granulomas (2). Crohn's disease conversely can involve any part of the GI tract, and also differs to UC in both its disease pattern, types of disease behaviour, and is more associated with the presence of granulomas on mucosal biopsy (3). Up to fifteen percent of IBD cannot be readily classified into either of these subtypes, and is termed IBD unclassified (IBDU). Indeterminate IBD is defined as IBD that remains unclassified after histological analysis of a post colectomy (4).

Since the discovery of ulcerative colitis in 1875 and Crohn's disease in 1931, the incidence of these conditions have been steadily rising in the western world including in Europe, the USA, and Australia, with a prevalence of 0.3% (5), and the incidence is rising towards Western levels in newly developed industrialised countries (6, 7). In the United Kingdom (UK), Crohn's disease has a prevalence of 157 per 100,000 people, equating to at least 115,000 people (8), and UC is slightly more common with a prevalence of approximately 240 per 100,000, equating to at least 146,000 people (9).

Compared to autoimmune conditions which show a higher prevalence in women, gender separation is less distinct in IBD. However, gender differences have been observed in clinical presentation, medical and surgical intervention, and disease course (10). Peak incidence of IBD

appears to between the second and fourth decades of life, and a smaller peak in the sixth decade (11), albeit this later peak has not been replicated in more recent studies (12). Ethnic background also influences disease phenotype, with Northern European Caucasians having a higher prevalence of stricturing and penetrating Crohn's disease compared to South Asians (13, 14), and a lower prevalence of extensive disease compared to patients from a South Asian background (14-16).

Obesity, a growing worldwide health concern, is increasingly of interest in research and clinical management of patients with inflammatory bowel disease. The prevalence of obesity in IBD is at least as common as it is in the general population (17), with 15–40% of patients with IBD being reported as obese (18). The effect of obesity on disease phenotype and activity has varied in the literature. A higher frequency of elevated CRP and lower IBD-related quality of life has been associated with obesity in one study (19), along with a shorter time to first surgery (20), and a shorter time to developing peri-anal complications (21). However, no differences in risk or number of IBD-related surgeries, IBD-related hospitalisations, and initiation of anti-TNF treatment have been also reported (17, 19, 20). A better disease course has also been reported, with lower rates of surgery, hospitalisation and treatment escalation to anti-TNF therapy (17), and a lower prevalence of penetrating disease in having been associated with obesity (22). Obesity is associated with a chronic low-grade inflammatory state (23), and potential differing disease course in IBD patients has been associated with adiposity related adipokine (adipocyte produced cytokines) dysregulation (24, 25).

The diagnosis of inflammatory bowel disease in general is made using clinical, biochemical, stool, endoscopic, histological, and radiological information (26). From a clinical perspective, patients can present with varying symptoms depending on sub-type of IBD, location or disease extent, disease behaviour, and severity of inflammation. Symptoms include diarrhoea, urgency

to defecate, per rectal bleeding, and abdominal pain. Constitutional symptoms can include weight loss, fever and fatigue. Crohn's disease, can have a much more heterogeneous presentation, and some patients may present with complications such as an intra-abdominal collection, bowel obstruction, or perianal disease.

Crohn's disease can affect any part of the GI tract, but typically affects the terminal ileum and colon, whereas ulcerative colitis only affects the colon, although some patients with pancolitis can get backwash ileitis. Unlike ulcerative colitis, Crohn's disease has different disease behaviours; as well as inflammatory, it can be stricturing and/or penetrating, the latter leading to intra-abdominal fistulisation and collections. Crohn's disease can also involve the ano-peritoneal area, leading to peri-anal complications including fistulisation and abscess formation.

From a histo-pathological perspective, Crohn's disease is a transmural disease with a classically patchy distribution, and ulcerative colitis is a continuous colonic mucosal disease. Both Crohn's disease and UC are characterised by chronic inflammatory changes, and in practice can be difficult to distinguish. Features favouring Crohn's disease include the presence of granulomas, patchy lamina propria inflammation, and focal segmental crypt distortion. Diffuse crypt abnormalities, crypt atrophy, abnormal crypt architecture, irregular mucosal surface and mucin depletion favour UC (27).

Accurate classification of IBD has been developed to assist in disease management and prognosis. The Montreal Classification system classifies Crohn's disease patients according to age, disease location, and disease behaviour. For UC, this classification system uses just disease extent (3). This classification system is summarised in the table 1.1.

	Crohn's disease	Ulcerative colitis
Age (A)	A1 below 16 y A2 between 17 and 40 y A3 above 40 y	-
Location (L) / Extent (E)	L1 ileal L2 colonic L3 ileocolonic L4 isolated upper disease*	E1 ulcerative proctitis - rectum only E2 left sided UC (distal UC) - inflammation distal to splenic flexure E3 extensive UC (pancolitis) - extends proximal to the splenic flexure
Behaviour (B)	B1 non-stricturing B2 stricturing B3 penetrating p perianal disease modifier†	-

Table 1.1: Montreal classification of Crohn's and ulcerative colitis. *a modifier which can be added to L1-3 when upper GI disease present, † is added to B1–B3 when concomitant perianal disease is present.

1.1.2 Pathogenesis of IBD – multiple component approach

The cause of IBD remains largely unknown (28), but there have been significant advances in our understanding over the past two decades (29). The consensus agreement is that genetic variations and environmental factors including changes in the intestinal microbiome lead to a chronic abnormal immune response (29-31). The pathogenesis of IBD is therefore a result of a 'network effect' of these components, and this effect is called the 'IBD interactome' (32, 33). Figure 1.1 summarises the component model of IBD pathogenesis.

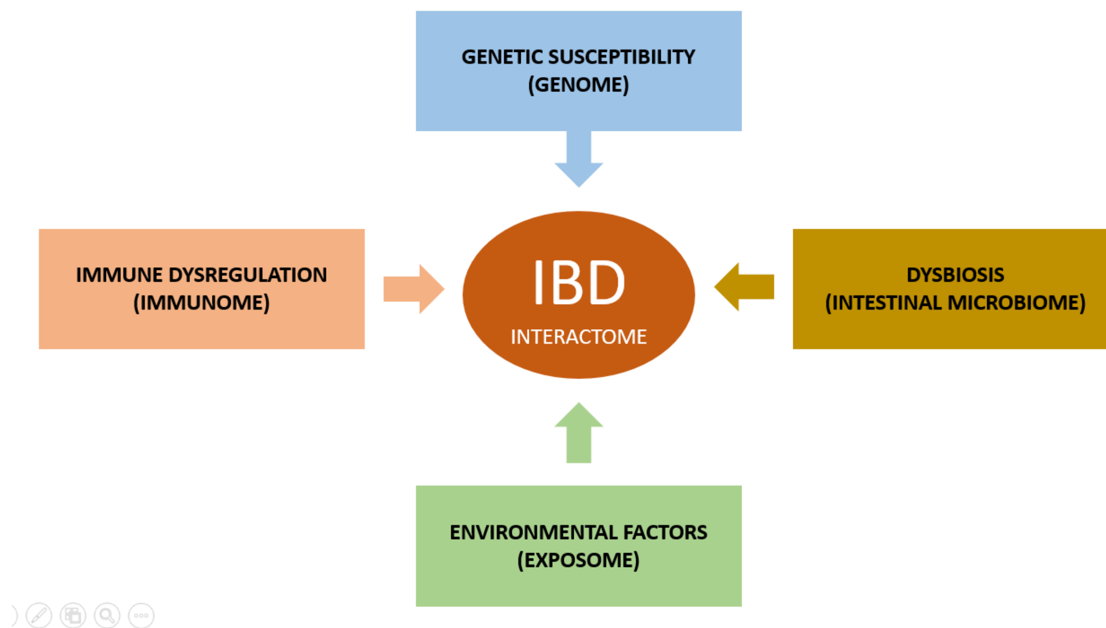

Figure 1.1: Component model of IBD pathogenesis

Research to date has been heavily focused on studying individual '-omes' in isolation. Integrated research into the multiple '-omes' will help build a comprehensive molecular map of IBD (29, 33). The aim will be that IBD molecular subtypes can then be identified that will correlate to clinical phenotypes which will help elucidate central molecular hubs which can be targeted by new drugs (33).

1.1.2.1 Genetics

There are over two hundred genetic variations already discovered that are strongly associated with IBD using genome-wide association studies (GWAS) (33). The more IBD-associated genes within an individual, the greater the risk of developing IBD (34). However, genetic variation alone cannot explain the aetiology of IBD, as it only accounts for up to a quarter of all IBD cases (29, 33). Monozygotic twin studies have shown concordance rates of 30-35% in

Crohn's disease, and 10-15% in ulcerative colitis, suggesting that non-genetic pathogenic components play an important role in IBD (31).

Studies have identified specific gene variants with plausible disease mechanisms, including the nucleotide-binding oligomerization domain-containing protein 2 / caspase recruitment domain-containing protein 15 (NOD2/CARD15) and autophagy-related 16 like 1 (ATG16L1) gene variants in Crohn's disease. Both have been linked to an inappropriate immune response and defective removal of intestinal microorganisms (30, 31, 33). Several gene variants are associated with specific disease phenotypes, including NOD2/CARD15 that has been specifically linked to ileal fibro-stenotic Crohn's disease (35).

Defective autophagy of bacterial cells has been associated with mucosal epithelial and dendritic cells containing ATG16L1 and NOD2/CARD15 variants (36). Autophagy, the destruction and recycling of cellular components, is an important process that controls bacterial replication and antigen presentation within the intestine (28, 36).

Other susceptibility gene loci include several related to the Th17 and IL-23 pathway, which includes the IL23R gene that encodes for the pro-inflammatory cytokine IL-23. JAK2, IL12B and STAT3 are also associated with this pathway, and these cytokines are associated with IBD (36). Overall, genomics can be beneficial in identifying subgroups of patients at risk of a specific disease phenotype, but as all genes are affected by epigenetic modification, and so genetic variants identified by GWAS will be have increased utility when combined with other pathogenic components such as DNA methylation (29, 30).

1.1.2.2 Immunology

Until recently the focus in IBD pathogenesis has been the altered immune response, and in particular, abnormalities in the adaptive immune system (33). A correlation has been observed

with Crohn's disease and a Th1 response, and a Th2 response with UC (28). More recently, the importance of the innate immune system has been recognised, which includes the integrity of the epithelial barrier, innate microbial detection, and autophagy (36). It has also been recognised that to take our understanding further, the immune response cannot be examined in isolation, and its interaction with the exposome and intestinal microbiome must be taken into account as immune homeostasis depends on the immune and non-immune cells interacting with the intestinal microbiota allowing reciprocal regulation (29).

Cell surface toll-like receptors mediate the gut innate immune response, along with cytoplasmic NOD-like receptors. Altered expression and function of immune cells with both TLRs and NOD proteins through mutations such as NOD2/CARD15 is suggested by evidence to date to lead to reduced activation of the inflammatory NF-κB pathway (37), which may cause an inadequate anti-bacterial response and subsequent pathogenic organism invasion. NOD2 loss of function mutations may also lead to an excessive Th1 response by inhibition of TLR2 stimulation (38). Altered bacterial autophagy from mutations in NOD2/CARD15 and ATG16L1 have also been associated with IBD (31).

IL23, along with its role in the adaptive immune response, can influence innate immunity (36). Polymorphisms in IL23R, associated with IBD, can affect Th17 cytokine function. Increased bacterial translocation and defective barrier function from the mucous and epithelial layers have been observed in IBD (29, 36). IBD has also been associated with defective secretion of antimicrobial peptides by the epithelial layer (36).

The abnormal adaptive immune response in Crohn's disease centres around the Th1 cell response (28, 29, 36), induced by IL-12, and leads to a production in IL-12. Mucosal T cells then elevate IFN-γ and IL-2, leading this abnormal Th1 response to cause intestinal inflammation (36). Conversely, UC is a Th2 adaptive response, as there are higher amounts of

IL-5 and IL-13, and low IL-4 (28, 29). Immuno-pathogenesis of IBD is also linked to Th17, and are characterised by secretion of IL-17A, IL-21, and IL-22. Clonal expansion of these cells is promoted by IL-23 (29, 36). High levels of IL-17A, one of the main cytokines associated with Th17 cells, has been found in the mucosa of IBD patients (29).

1.1.2.3 Environmental factors

The exposome refers to all external factors that humans are exposed too. The intestinal microbiome is also part of the exposome (29), but as it is located inside the human intestine and has received such recent interest in IBD pathogenesis, it will be addressed separately. The importance of the exposome has been highlighted by two main factors. The first, is the increase in incidence and prevalence in the developed countries during the second half of the last century. This time scale alone would not allow for a significant change in the human genome to explain this rise (39). The second factor is the effect in incidence of IBD in migrants who move from areas of low IBD incidence to areas of high incidence. The infants and second generation of these migrants have a similar incidence of IBD as the host country, suggesting that environmental factors as opposed to genetics play a more important role in this rise in incidence (40).

Several external factors have been linked to IBD, with evidence linking smoking, a few drugs, vitamin D, diet, stress, air pollution, education status, and appendicectomy to disease onset and changes in disease course (28, 29, 36). Heavy smoking confers a protective effect in the onset and relapse of ulcerative colitis, and is associated with a higher risk of disease onset and surgery in Crohn's disease. A higher risk of IBD has been correlated with low vitamin D (41), and in mouse studies (42), has been shown to ameliorate severe intestinal inflammation. Non-steroidal anti-inflammatory drug (NSAID) long-term exposure has also been associated with an increased risk of IBD (43, 44), and along with antibiotic use (45). Stress has been linked to

both the onset of IBD, and as a mediator of disease exacerbations (46). Air pollution may also have a role, with recent evidence showing an association with rising incidence of IBD and industrialisation in developing countries (36, 39).

Diet has a significant impact on developing IBD, with high-sugar, high-fat Western diets associated with an increase in incidence and prevalence of IBD (29). Evidence has shown a link between diet and the intestinal microbiome, the latter have an established central role in the pathogenesis of IBD (47). Babies are born with a sterile gut, and breast or formula milk, and then exposure to other food, is integral to the development of the intestinal microbiome (48). A study has linked different dietary patterns as the reason for children from rural Africa having a different abundance of the main bacterial phyla (*Bacteroidetes* and *Firmicutes*) compared to children living in Europe (49).

1.1.2.4 Gut microbiome and dysbiosis

1.1.2.4.1 Role of intestinal microbiome in health and disease

The intestinal microbiome, defined as the entire habit of microorganisms, their genomes, and their surrounding environmental conditions within the intestine (50), comprises of approximately 10^{13}-10^{14} bacteria, and over a 1000 species (51). Each person has around 160 species of bacteria, along with viruses, protozoa and fungi (51, 52). The composition and abundance of bacteria varies within different parts of the intestine (53). There are two main functional phyla, *Bacteroidetes* and *Firmicutes*, although a further 10 can have important function on the GI tract (54, 55). The ratio of *Firmicutes*:*Bacteroidetes* also varies within individuals when studied in large cohorts (52).

The intestinal microbiome starts to develop immediately following birth, and is relatively established within the first few weeks of life (28, 29). During this developmental period, the

microbiota is thought to be critical in priming the host intestinal mucosal immune system, and disruption at this stage could move the gut away from being healthy, and to being more prone to conditions such as IBD (29). Following development in childhood, the microbiome reaches stability in adulthood, and then declines later in life (56, 57). Once established in adulthood, there is evidence to show that each individual's microbiota remains relatively stable, and has some resistance to long term changes caused by diet, drugs, pre- and probiotics, surgery and infections (57).

The microbiome contributes to the development and regulation of the immune system, digestion which includes synthesis of essential vitamins and short chained fatty acids, drug metabolism, and protection against pathogens (51, 54, 58). It therefore plays an important role in health, and so significant perturbations in the intestinal microbiome can affect the multitude of host immune-microbiota interactions, and potentially lead to and perpetuate chronic inflammation (51, 58-60). This compositional as well as functional disturbance in the enteric microbiota is termed dysbiosis (52).

1.1.2.4.2 Dysbiosis and IBD

The human enteric microbiome is thought to have a crucial role in the pathogenesis of inflammatory bowel disease. Dysbiosis in IBD is characterised in general by a loss of anti-inflammatory microbiota, and an increase in pro-inflammatory microbiota (51), resulting in an overall 'loss of tolerance' of host enteric immunity to the resident microbiota (33) with a disruption of microbiota-microbiota, and microbiota-host cross-talk (61).

There has been some debate in the literature as to whether dysbiosis is the cause or result of IBD (62). There is evidence to support a causal role in IBD in both animal and human studies. Multiple murine studies in genetically susceptible germ-free individuals have shown a rapid immune response and subsequent inflammation in response to introducing microbiota, and

particularly when transferred from mice with established colitis (62-65). A study by Schaubeck et al. (66) demonstrated transmission of Crohn's disease-like ileitis in susceptible murine hosts using inflammatory-associated microbiota compared to non-inflammatory controls. A human study by Rutgeerts et al. (67) showed that patients who underwent diversion of the faecal stream by defunctioning the small bowel proximal to the site of curative ileal resection had no disease recurrence compared to the control group that had a one-step resection and anastomosis, and once these defunctioned patients were re-anastomosed, disease recurred in all. Similarly, infusing small bowel luminal contents proximal to an ileocolonic anastomosis via a defunctioning loop ileostomy following curative bowel resection for Crohn's disease was shown to initiate inflammation in a study by D'Haens et al. (68) These studies have all shown that introducing intestinal microbiota in a susceptible intestine triggered inflammation, suggesting a role of the microbiome in triggering inflammatory bowel disease. Treating active Crohn's disease with antibiotics has been shown to have a clinical response, and in some patients lead to clinical remission, again implicating the microbiome in the aetiology of IBD (69).

There is also evidence suggesting dysbiosis is also the consequence of inflammation (70). Changes in IBD activity have been associated with changes in the microbiome. Results from human studies have been inconsistent, and in some cases contradictory, and this is likely due to high heterogeneity in human subjects (71). However, findings from animal studies have been far more reproducible, and studies have shown changes in the microbiome in the DSS colitis induced mouse model during and following repeated episodes of inflammation compared to intervals of no inflammation and controls (71-73).

There is strong evidence that a reduction in microbial diversity is associated with IBD - the healthier and more diverse the microbiome, the more effectively it interacts and regulates the

host immune system, and vice-versa (29, 74). Along with the effects on diversity, changes in composition has been reported in a consensus bacterial profile for IBD which was established as the result of more than 600 studies of the gut microbiota in this disease (51). *Proteobacteria* (particularly adherent-invasive *Escherichia coli*), *Fusobacterium*, *Veillonellaceae*, *Pasteurellaceae*, and *Ruminococcus gnavus* are taxa that are commonly reported as higher in abundance in IBD (70). *Clostridium* groups *IV* and *XIVa*, *Faecalibacterium prausnitzii*, *Bifidobacterium*, *Bacteroides*, *Suterella*, and *Roseburia* are typically lower (52, 70). At phyla level, study findings have been much less consistent, but most report an increase in *Bacteroidetes* and *Gammaproteobacteria*, (75, 76), and several studies have shown a loss of *Firmicutes* (77). Other microorganisms have shown to differ in abundance through emerging research. Some *Candida* species are higher in IBD including *Candida albicans* and *Candida tropicalis*, as well as lower levels of the fungus *Saccharomyces cerevisiae* (78). *Caudovirales*, which are bacteriophage viruses, are reportedly higher in IBD (79).

1.1.2.4.3 The gut microbiome and the IBD interactome

Our understanding of the human intestinal microbiome has increased enormously over the last two decades (33, 52), along with our understanding of changes in the gut microbial composition associated with IBD (51, 59, 62, 80), other GI conditions (81, 82), and some non-GI conditions including asthma, diabetes, heart disease, neurological diseases and cancer (83-86). However, compositional changes are not enough to translate these findings into potential new therapeutic targets (29, 30, 52). To do this, better understanding of how the gut microbiota interacts with host physiology will be needed (52), as the pathogenesis of IBD is a result of a ‘network effect’ of different ‘-omes’ - the ‘IBD interactome’ (33). Therefore, further integration of changes in the gut microbiota with other 'omics', including genomics, epigenetics, proteomics and metabonomics will be needed to help build a comprehensive

molecular map of IBD, allowing individualised therapeutic targets to form more personalised healthcare in this condition (32).

Research integrating genomics and the gut microbiome includes identification of several susceptibility genes linking microbial sensors that activate autophagy such as NOD2/CARD15, and genes that are linked to regulation of autophagy such as ATG16L1 (33, 87). Disruption of these proteins leads to defective microbial clearance which has been associated with Crohn's disease (87).

Metabolic profiling, termed 'metabonomics' or 'metabolomics', is a powerful approach to linking cellular pathways to biological mechanisms (88), and can help take forward our comprehension of the intestinal microbiome function, and how the developmental and regulatory dysfunctional between host and microbiome relates to the pathogenesis of IBD (29, 30, 32). Metabolites are small molecules that provide a functional readout of cellular biochemistry, and unlike genes are not directly influenced by epigenetic regulation and post-translational modifications, and so act as direct signatures of biochemical activity (88). Using spectrometry techniques, thousands of metabolites from biological samples can undergo qualitive and quantitative measurement, and then can be integrated with other 'omic' techniques (30, 32).

Alterations in microbial composition in IBD have been well described, along with the importance of colonic metabolites in signalling and immune system modulation. There have been recent advances in how bacteria and the metabolites they modulate contribute to IBD (62, 80, 89). A recent study by Franzosa et al (89) was one of the first to examine gut metabonomic and microbiomic changes associated with IBD in an integrated multi-omic framework. It concluded the discovery of possible mechanistic relationships between specific metabolites and microbes that are perturbed in IBD, with over 100 robust associations found between well-

characterized differentially abundant metabolites and differentially abundant species. Another study (58) exploring the functional aspect of the IBD microbiome using metabolic profiling showed that carbohydrate-related metabolic pathways and production of amino acid were decreased by changes in the enteric microbiome associated with IBD.

Several other bacterial metabolic pathways have been identified through integrating microbial analysis with metabonomics, including the identification of the nuclear farnesoid-activated X receptor (FXR) pathway, which is a bile acid receptor involved in bile acid signalling (62). This has shown that dysbiosis could affect FXR signalling as intestinal bacteria bile salt hydrolases (BSH) are integral bile acid biosynthesis (62, 90). Silico analysis has shown that there is a marked reduction in BSH activity in particularly the *Firmicutes* phyla of Crohn's patients compared to healthy control (91). There is also a loss of secondary bile acids in patients with IBD consistent with impaired microbial enzyme activity, leading to modified metabolism of bile salts metabolism and through the FXR pathway a subsequent reduction of anti-inflammatory signalling (92).

Short-chain fatty acids (SCFAs) produced by specific *Clostridia* species through the fermentation of undigestible carbohydrates increase intestinal regulatory T-cell function by promoting immune tolerance (93), and this has been shown to reduce colitis in murine studies (94). Studies are currently underway to target parts of these IBD microbial pathways, either through novel small molecule drugs or by directly altering the gut microbiota (62).

1.1.2.4.4 Techniques to investigate the gut microbiota

Next generation sequencing (NGS) techniques have revolutionised exploration of the human intestinal microbiota, identifying over 99% of the microbiota compared to previous culturing techniques that could only identify about 30% of species present (95). Development of DNA sequencing and computational analysis allowed multiple research groups to examine the

human gut microbiota using small-subunit (16S) ribosomal RNA gene-sequence-based experiments (16S rRNA sequencing). The 16S rRNA gene, found in all microorganisms, is conserved sufficiently to allow precise phylogenetic analysis (54). This technique allows for accurate survey of bacterial communities within biofluids and tissue.

Shotgun metagenomics is another next generation sequencing technique that performs untargeted sequencing of all the microbial genomes within a sample, and not just the 16S gene (96). It can therefore be used to profile taxanomic composition and functional potential of microbes.

Metaproteomics is a technique used to give direct information about bacterial gene function. Shotgun metaproteomics uses untargeted mass spectrometry to measure thousands of proteins within a faecal sample, which can then be correlated to metagenomic analysis to give information about which genes are being expressed (97, 98)

1.1.3 Treatment and the future of personalised healthcare

IBD is a long-term condition that generally follows a relapsing and remitting course, and often affects people younger in life. It can have significant effects on quality of life, participation in the workplace including time off work, educational performance, and there is a small increase in mortality (99). Medical therapy currently aims to induce clinical remission, achieve mucosal healing, and prevent complications including hospitalisation, surgery, and cancer (100). From the early days of just steroid based treatment for UC in the 1950's, there are now a large array of treatments including 5-aminosalicylates, oral immunomodulators, and manufactured antibodies and small molecule therapies targeting specific aspects of the immune-mediated inflammatory response (26, 101) – see Figure 1.2. There remains, however, considerable risks and adverse reactions associated with IBD treatment, and in light of phenotypic heterogeneity,

finding the optimum treatment course for individual patients can be timely and challenging (101).

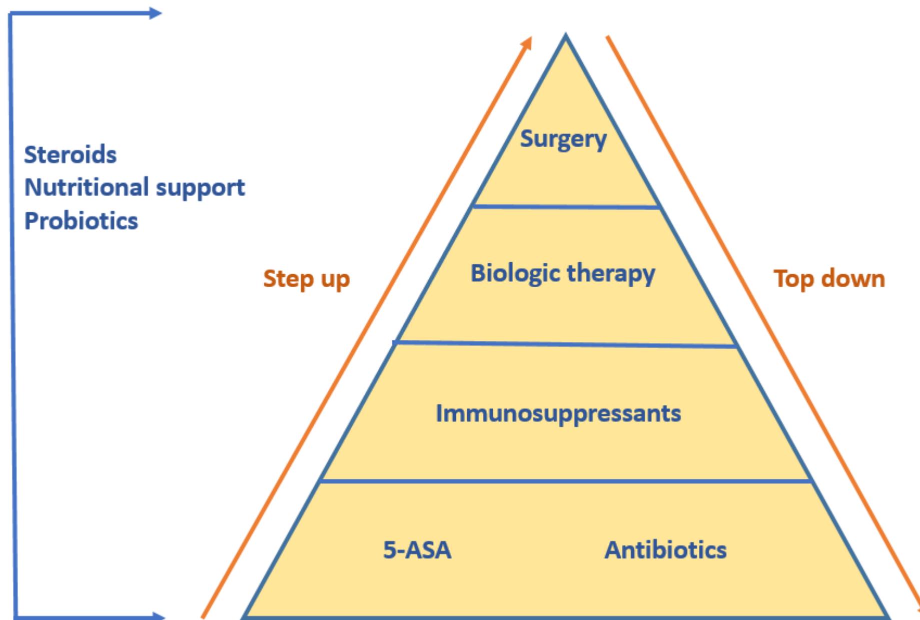

Figure 1.2: Treatment pyramid for IBD.
Adapted from figure published by University of Alberta IBD Clinic website (102)

Medical treatment of inflammatory bowel disease depends on several factors, including disease subtype, location, extent, behaviour (in Crohn's disease), and complications. The mainstay of medical therapy in ulcerative colitis is 5-aminosalycilate (5-ASA) based treatment, either oral and/or topically administered. 5-ASA treatment is less effective in Crohn's disease, and the most recent BSG IBD guidelines published in 2019 has concluded there is no role for mesalazine in Crohn's disease, including disease confined to the colon (103). In contrast, non-systemic steroids and oral immunomodulators such as azathioprine have better reported efficacy (104). For severe disease in both Crohn's disease and UC, systemic steroid therapy with either oral prednisolone or intravenous hydrocortisone is often used (104). Biological therapy using anti-TNF based treatment with infliximab and then later adalimumab have been

used for severe or treatment refractory IBD over the last two decades (105). There has been an introduction of several newer biologic classes for the treatment of IBD over the last 5 years - anti-α4β7 integrin and anti-IL12/23 antibody treatment. The most recent novel drug treatment introduced are the Janus kinase (JAK) inhibitors, which are small molecule drugs rather than biological antibody treatment for the treatment of ulcerative colitis (106, 107).

5-aminosalicylate based therapy is principally used for mild to moderate ulcerative colitis (104), and in one study assessing treatment response in mild to moderate UC (108), high dose oral 5-ASA monotherapy was shown to achieve remission in 43% of patients at week 8, and 64% of patients in combination with topical treatment. The mechanism of action is not fully known, but these drugs may act by having a direct anti-inflammatory effect on the mucosa by reducing leukotriene and prostaglandin production through inhibition of cyclooxygenase and lipoxygenase pathways. This in turn is thought to block peptide-induced neutrophil chemotaxis, reduce scavenging reactive oxygen metabolites, and possibly prevent activation of nuclear factor-κB (109, 110). Oral 5-ASA is combined with one of two main delivery systems to allow it to take effect in the distal ileum or colon – either being coated in a pH sensitive resin or a semipermeable membrane, or being linked with another molecule by an azo bond (110). These drugs are generally well tolerated, with interstitial nephritis being the most important but rare side effect (111).

Steroids have long been an effective treatment in inducing remission in Crohn’s disease and UC patients (104, 111). In Crohn’s disease, two major studies have shown the benefit of systemic steroids in active disease (104), with the first study (112) showing 60% of patients achieving remission (number needed to treat [NNT] = 3) with a tapering prednisolone regime, and the second study (113) showing 83% achieving remission with 6-methylprednisolone (NNT = 2). Non-systemic steroid therapy with oral budesonide has also shown benefit in active

ileal and ileocolonic Crohn's disease, but with less efficacy (114). In UC, systemic steroids are indicated in moderate and severe disease, or mild disease that does not respond to 5-aminosalicyate based treatment. Non-systemic steroid treatment is indicated in mild to moderate UC refractory to 5-ASA treatment (111). The wide adverse effect profile has long been the concern with steroid treatment, with early onset adverse effects including mood and sleep disturbance, cosmetic issues including weight gain, and glucose intolerance. Serious effects associated with long term use of steroids include osteoporosis, necrosis of the femoral head, myopathies, and increase rates of infections (104).

Thiopurines form the mainstay of oral immunomodulator treatment in Crohn's disease and UC, and are used principally as maintenance treatment in light of their slow onset of action. There is substantial low-quality evidence demonstrating the efficacy of azathioprine as a maintenance treatment in Crohn's disease, with one pooled analysis of six studies showing 73% of patients remaining in remission over a 6 to 18 month follow up period (115). In UC, pooled analysis of seven studies showed that 44% of patients on Azathioprine failed to maintain remission compared to 65% of placebo patients (116). Increasingly thiopurines, along with other oral immunomodulators such as methotrexate if not tolerated or contraindicated, are used in combination with anti-TNF treatment to prevent immunogenicity of this class of biological treatment (117).

Thiopurines inhibit ribonucleotide synthesis causing T-cell apoptosis and changes in T-cell subpopulations, the former by modulating Rac1 signalling within the cell. Azathioprine is metabolised to 6-mercaptopurine (6-MP), and then to 6-thioguanine nucleotides (6-TGN) which is the active metabolite (104, 118). Approximately 20% of patients do not tolerate thiopurines, and serious side effect include infections, acute pancreatitis and lymphoma (118).

Other oral immunomodulators used in IBD include methotrexate, tacrolimus, and mycophenolate (119).

The first class of biological therapies used in IBD were IgG1 anti-TNF monoclonal antibodies, with Infliximab being the first drug licensed in the UK. Its efficacy was shown in a multicentre, double-blind study by Targan et al (120), where 64% of Crohn's disease subjects with moderate to severe disease activity that was not responsive to 5-ASAs and steroids showed a clinical response compared to 17% who were given placebo (NNT = 1.6). A study by Schnitzler et al (121) showed that 89% of patients achieved clinical response after induction therapy. Adalimumab was the next anti-TNF drug to be licensed for Crohn's disease, and was shown in the CLASSIC 1 study (122) to have a superior affect compared to placebo in the treatment of anti-TNF naïve Crohn's disease patients with moderate to severe disease activity, with clinical remission being achieved in 36% of patients at week 4 compared to 12% given placebo.

The efficacy of infliximab has been demonstrated as a rescue treatment for acute severe colitis, with one study (123) showing a significant reduction in colectomies at 3 months. The ACT-2 trial (124) showed the benefit of infliximab in steroid refractory UC patients, with 21.5% of patients with endoscopically confirmed moderate to severe disease achieving steroid free remission at 30 weeks compared to 7.2% who received placebo. The ULTRA-2 trial (125) was a pivotal study for adalimumab, and showed that 31% of steroid refractory patients with UC had ceased steroid treatment at week 16 compared to 5.7% of patients receiving placebo. The SONIC trial (105) showed that combination therapy of Infliximab and Azathioprine was superior to infliximab or azathioprine alone. Similarly, the SUCCESS trial (126) showed benefit of combination therapy compared to Infliximab or Azathioprine monotherapy in ulcerative colitis.

Serious adverse effects of anti-TNF treatment include infections, reactivation of latent tuberculosis, and an increased risk of malignancy (104). A meta-analysis in 2009 showed an increased risk of lymphoma with anti-TNF treatment in IBD compared to thiopurines (127).

Newer classes of biological therapies include anti-migratory therapies such as the α4β7 integrin inhibitor vedolizumab. In the GEMINI-III trial (128) for moderate to severely active Crohn's disease with previous treatment failure to anti-TNF therapy, 15% of patients achieved clinical remission at 6 weeks compared to 7% of placebo. In UC, the GEMINI-I trial (129) showed that at week six 47.1% of patients receiving vedolizumab had responded compared to 25.5% of placebo. A head-to-head study showed that vedolizumab was superior to adalimumab in acquiring clinical remission in the treatment of ulcerative colitis (130).

Ustekinumab, that targets IL-12 and IL-23 receptors, was studied in the UNITI trials and has been licensed in moderate to severely Crohn's disease, and has shown significantly higher clinical response rates at week 6 compared to placebo (UNITI-2, 55.5%, and 28.7%, respectively) in anti-TNF naïve or previous response groups, as well as in anti-TNF refractory patients (UNITI-1, 34.3% and 21.5%, respectively). At week 44 (IM-UNITI), remission was achieved in 53.1% of patients on 8 weekly dosing compared to 48.8% on 12 weekly dosing and 35.9% in the placebo group. At this time period, serious adverse events occurred in 9.9% on 12 weekly dosing, 12.1% on 8 weekly dosing, and 15.0% in the placebo group, with serious infection in 5.3%, 2.3%, and 2.3% respectively. (131). In ulcerative colitis, ustekinumab has been shown to be superior to placebo in achieving clinical remission through the UNIFI trials in patients with prior biologic exposure (either non-responder or intolerant), with clinical remission achieved following induction (week 8) in 15.6% of the treatment arm compared to 5.3% in placebo arm, and clinical remission in the maintenance part of the study (week 44) with 43.8% receiving 8 weekly treatment compared to 24.0% in the placebo group. Safety data

for UC patients receiving ustekinumab was reported at 52 weeks as 7.6% on 12 weekly dosing, 8.5% on 8 weekly dosing, and 9.7% receiving placebo, and with regards to serious infections, 3.5%, 1.7%, and 2.3%, respectively (132).

Another emerging biologic, risankizumab, which is currently used in the treatment of psoriatic arthritis, has been shown to be effective and safe in its phase 3 study for Crohn's disease (133). Risankizumab inhibits IL-23/p19 pathway, and has a reported clinical remission rate of 45% at week 12, compared to 22% with placebo.

The first oral small molecule therapy to be licensed in the UK was tofacitinib, a non-selective Janus kinase (JAK) inhibitor, where following induction (week 8, OCTAVE Induction 1 trial) clinical remission was achieved in 18.5% of patients receiving treatment compared to 8.2% in the placebo group (107). Patients included in this study had to have had prior treatment failure or intolerance in one of the following IBD treatments: glucocorticoids, thiopurines, or anti-TNF treatment. The OCTAVE Sustain trial (107) showed clinical remission (52 weeks) in 40.6% of patients compared to 11.1% in the placebo group. Adverse effects include higher rates of infections, skin cancer, lipid levels, and pulmonary embolism in patients with risk factors for thromboembolic disease (134).

Filgotinib, a selective JAK inhibitor (preferential to JAK 1), has induced clinical remission in 26.1% patients with ulcerative colitis compared to 15.3% in the placebo group in patients who were biologic-naïve, and 11.5% compared to 4.2% respectively in patients with prior biologic exposure (135). Upadacitinib, the latest JAK inhibitor (also preferential to JAK 1), has had positive efficacy and safety data reported for the treatment of ulcerative colitis in its phase 3 study published recently (136), and encouraging phase 2 data for Crohn's disease (137). This drug also has the additional benefit of efficacy in the treatment of overlapping inflammatory joint disease (138, 139). In October 2022 the European Medicines Agency announced safety

recommendations for the use of Janus kinase inhibitors for inflammatory conditions, advising there use in patients 65 years or older only if no other treatment options were available, and this is in light of concerns over infections, cardiovascular events, venous thromboembolic events, and cancer (140).

Ozanimod, another oral small molecule drug that selectively modulates the sphingosine-1-phosphate receptor, and is used in the treatment multiple sclerosis, has been shown to be effective in the induction and maintenance treatment of ulcerative colitis. Its safety data is also encouraging from the phase 3 trial, with serious infection at 52 weeks occurring in less than 2% of patients (141). Other novel compounds undergoing trials include SMAD7 antisense oligonucleotides, anti-IL-6 antibodies, and anti-Madcam antibodies (104).

Exclusive enteral nutrition (EEN), which comprise of a completely liquid diet, have increasing evidence for inducing remission in adults (142-146), albeit studies are have been small, despite previous large meta-analysis showing only significant benefit in paediatric cohorts (147, 148). EEN can be used as alternative to systemic steroids to induce remission, and are particularly useful when glucocorticoid treatment is contra-indicated, or in patients who have had previous significant adverse effects.

Although Crohn's disease is now managed primarily by medical gastroenterologists, surgery still plays an important role with many patients still undergoing surgery (149). Patients with ileo-caecal Crohn's disease carry a 90% risk of requiring surgery (150), although recurrent resections are no longer necessary and considered harmful for patients (149). Surgery in UC has been refined over the last 20 years, where the gold standard has moved away from procto-colectomy with ileostomy, to restorative procto-colectomy with an ileal pouch-anal anastomosis [IPAA] which preserves rectal tone and body image (151, 152). The rate of

colectomy in UC has decreased over the last two decades, with a recent study showing a 10-year risk of colectomy at 6.4% (153).

Current treatment models in IBD are based on disease classification, severity, patient factors including comorbidities and preferences, and following the step up or top-down model (see Figure 1.2). However, poor response to specific treatments can be high in IBD using the current approach, causing a considerable reduction in quality of life for patients, and have high cost implications for healthcare (152).

Most medical treatments for IBD have been developed to treat dysfunction within the immunome, which accounts for approximately 180,000 protein interactions (154). It is becoming increasing apparent that effective treatment of complex diseases cannot be done by modulating single targets (33). Current treatment approaches focusing on the immunome alone may have reached their limits (32). Within the IBD interactome, there are still many unknown interactions within and between each of the pathological components, including the genome, epigenome, proteome, metabolome and exposome (155). Each of these components contain at least hundreds of elements leading to a staggering number of potential interactions (32). Therefore, an integrated systems biology-based approach, along with classical hypothesis-led biology, is required to map out the disease network, and central hubs within it, to develop new targets to significantly advance individual treatment and develop personalised healthcare (30, 32, 33).

1.2 Metabonomics and Inflammatory Bowel Disease

1.2.1 Metabonomic analysis of human biofluids

Metabonomics, now used interchangeably 'metabolomics' and 'metabolic profiling' (156), encompasses any technique that can identify and quantify small molecules within a biological

sample (157). These techniques enable thousands of metabolites, the functional readouts of cellular biochemistry, to be quantitatively measured allowing new discoveries to link cellular pathways to biological mechanisms (157-159). The two most broadly used techniques are nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy (159). These platforms can identify compounds including organic acids, amino acids, sugars, lipids, phenolic compounds, and nucleosides (158).

Metabonomic analysis can be divided into targeted and untargeted analysis – untargeted analysis aims to identify a metabolic "fingerprint" of a combination of metabolites from the total complement of metabolites, whereas targeted analysis identifies and quantifies specific metabolites of interest within a sample (160).

NMR spectroscopy is highly automated, provides relatively rapid high throughput analysis, and has excellent reproducibility making it ideal for large scale experiments compared with gas and liquid chromatography–mass spectrometry (GC-MS and LC-MS).

^{1}H-NMR spectroscopy detects hydrogen-containing molecules within a sample to produce an NMR spectrum – which can then be analysed to identify and provide relative quantification of all the metabolites within a sample (159). ^{1}H-NMR spectroscopy can be applied to different biological samples including serum, urine, faecal extracts, saliva, breast milk and tissue.

NMR spectroscopy is sensitive to minor internal and external perturbations, and so can provide valuable information including environmental factors that other '-omics' techniques cannot identify. NMR requires comparatively little sample preparation (161), is non-destructive to the sample (unlike mass spectroscopy), and results are easily reproducible. The main disadvantage is that in light of its sensitivity to external factors, it is potentially subject to many confounders that need to be taken into account by acquiring detailed metadata including diet and medications, to allow results to be translated in a meaningful way. NMR spectroscopy are less

sensitive than mass spectroscopy, with NMR studies typically identifying 50–200 metabolites with concentrations >1 μM, whereas LC-MS can identify over a thousand metabolites with concentrations of >10 to 100 nM (162).

1.2.2 Application of metabonomics in clinical practice

In the 21st century there is a move to take medicine forward by treating each patient as an individual interactome, with unique physiological, biochemical and environmental interactions. Therefore, metabonomics, with its ability to analyse metabolites representing the products of complex host-environmental interactions, presents itself as an ideal area to help take personalised healthcare forward (163).

Within the field of oncology, translational use of metabonomics is further ahead compared to other fields, and can broadly be divided into imaging-based applications and using biofluid markers to guide treatment (164). In vivo studies of intra-operative rapid ionisation mass spectrometry (REIMS), known as the "iKnife", can distinguish between malignant and normal tissue in near real-time to assist the surgeon minimising excessive resection of normal tissue. The technique involved a diathermy cutting tool generating gaseous-phase ionized cell constructs that are suctioned into a time-of-flight mass spectrometry where metabolic profiles are compared to histologically-specific mass spectral libraries (165-167).

Metabolic profiling of biofluids have been demonstrated to discriminate cancer subjects from controls, which has included tumours of breast, lung, colorectal and pelvic origin (168-173). Furthermore, metabonomics has potential in assessing treatment response, with NMR spectroscopy identifying metabolic signatures from human glioma cells that are more likely to respond to chemotherapy, and can prognosticate prior to imaging detection, with better chemotherapy outcomes in several different cancers associated with lower total choline signals

(174). In breast cancer, serum metabolite profiles have been associated with micro-metastases and along with treatment outcomes (175, 176).

Non-oncological diseases have shown potential in utilising metabonomic techniques. A case series by Wang et al. (177) using mass spectrometry measuring serum 2-aminoadipic acid (2-AAA) levels, could predict a greater than four-fold risk of diabetes in subjects with the highest concentrations of 2-AAA. In rheumatology, gas chromatography/time-of-flight mass spectrometry analysis of synovial fluid has identified 20 metabolites than can distinguish rheumatoid arthritis from other inflammatory conditions (178).

Serum metabonomics has demonstrated a role in the stratification of neurological conditions, including multiple sclerosis and traumatic brain injury. A study by Villoslada at al. (179) identified metabolic signatures in longitudinal serum samples which could discriminate between controls, medium disability and high disability related to this disease. Serum analysis has also been shown to predict outcome in patients following traumatic brain injury, with relative quantification of medium chain fatty acids and one sugar derivative being closely associated with outcome severity (180).

Potential clinical use of metabonomics have been identified in cardiovascular and pulmonary diseases. Urinary NMR spectroscopy has identified markers including formate and hippurate that correlate with lung function in patients with COPD (181). Serum and blood sample metabolites have also been associated with the development of atherosclerosis reported in several studies, indicating a potential role as a population screening tool for heart disease (182-185).

The application of metabonomics in inflammatory bowel disease is discussed in the next sub-chapter, however, translating these techniques to widespread clinical practice has been prevented by several issues. Intra-operative near real-time histological assessment of tumour

margins using the metabonomic techniques have been affected by high start-up costs, and need to develop local expertise (165-167). For metabonomics applied to biofluids, as well as the above, several areas have been identified that require addressing before this field can be applied widely to clinical practice (163).

Firstly, there has been significant variation in how samples are collected and handled, sample pre-processing, and overall experimental procedures, and therefore standardisation to ensure reliable and reproducible results is needed - the National Phenome Centre based at Imperial College London has taken on this service in the UK (163, 186).

Analysing data and applying it in a standardised fashion for metabonomics to be reproducible for widespread use is another significant hurdle, particularly as different analytical platforms are used, which comprise of mass spectrometry techniques and NMR spectroscopy (163, 186-188) – these are discussed in more detail later in this thesis. Unlike with intra-operative tissue margin techniques, biofluid metabonomics produce non-binary results where the metabolic profiles will vary due to physiological and environmental factors (such as diet and medication). Development of local and national infrastructure, training local expertise, and having a national databases to aid interpretation of results will all be required (163).

1.2.3 Metabonomic profiling in IBD

There have been multiple metabonomic studies in IBD conducted over the last 15 years, with the initial aim of identifying biomarkers for IBD to help differentiate this condition from other GI disorders, and also Crohn's disease (CD) from ulcerative colitis (UC) in the 10-15% of patients with IBD unclassified. Over this time period, a single biomarker or panel of biomarkers (an 'IBD metabolic profile') has not translated from bench to bedside, and this is despite multiple studies being successful in using metabolic profiling to separate IBD from controls, and Crohn's disease from UC. Part of the issue has been reproducibility of results,

likely due to different study methodologies, sample handling and protocols, analytic platforms, and addressing of confounding factors (as described in the above section). Despite this, promising results from several studies has shown a potential role of metabonomics in assessing patients' disease activity and response to treatment.

Several studies have been able to discriminate active and quiescent disease, using different biological samples and analytical platforms, and correlating metabolic signatures with different assessments of disease activity used in current clinical practice (189). A study by Keshteli et al. (190) assessed the urinary metabolome in patients who had undergone ileocolonic resection and were able to correlate metabolic signatures with post-operative Rutgeert's scores. Two studies have shown correlation with faecal metabolites and faecal calprotectin results in paediatric Crohn's disease cohorts (191, 192), and serum and plasma metabonomic studies have correlated disease activity for both Crohn's disease and UC respectively (193, 194). Breath metabolic profiling has shown correlation with symptoms and endoscopic activity score in UC (195), and HBI score, CRP and faecal calprotectin in Crohn's disease (196). Biopsied colonic tissue analysed using mass spectrometry can differentiate treatment-naïve, newly diagnosed, and deep remission UC patients in a study reported by Diab et al. (197).

Stratify treatment response using metabonomics has been reported in several studies to date (189). A study by Ding et al. (198) prospectively recruited Crohn's disease patients prior to commencing anti-TNF treatment, and collected urine, blood and faecal samples at baseline and several intervals within the first 18 months of treatment. Models could be generated to predict treatment response based on metabolic profiling in all three types of biological samples. Another longitudinal study by Aden et al. (199) collected faecal samples in patients receiving anti-TNF and anti-α4β7-integrin (vedolizumab) therapy, and were able to generate predictive models in patients receiving anti-TNF treatment showing the potential of metabonomics to

stratify patients into those more likely to achieve clinical remission. The anti-α4β7-integrin cohort was underpowered and so this could not be assessed. Faecal metabonomic studies have shown promising results in predicting response to exclusive enteral nutrition (EEN) in Crohn's disease (200, 201), and potentially in faecal microbiota transplantation in UC (202, 203).

Overall, research to date has been promising in terms of metabonomics having a role in assessing disease activity and stratifying patients into treatment response and failure groups, but further work with larger longitudinal data, and likely integrating with other 'omic' data to identify molecular phenotypes will be needed to help predict clinical course and response to specific treatments more reliably.

As described above, a variety of biofluids have been used in IBD related metabonomic research, along with biopsy tissue samples taken at lower GI endoscopy. Biofluids include serum/plasma, faecal water and urine. The advantage of urine as a biological sample is that it is non-invasive, and easily obtained as patients can usually provide a sample on demand. Faecal extracts are also non-invasive obtain, but often cannot be produced on demand, and less acceptable to patients. Serum/plasma and colonic tissue are more invasive to acquire.

Animal studies, where the significant influence of confounders can be controlled for much more than in human studies, have shown that in urine the majority of distinguishing metabolites between subjects with colitis and controls are intestinal bacterial related (204). In serum, glucose and tricarboxylic acid cycle (TCA) cycle intermediates formed the predominant distinguishing metabolites (205).

Human studies assessing the role of metabonomics in IBD using colon biopsies and serum/plasma (not studied in this thesis) have found discriminatory metabolites associated with amino acid metabolism and the tricarboxylic acid cycle (204, 206, 207).

Most metabonomic studies have separated IBD cohorts into Crohn's disease and ulcerative colitis, and excluded patients with unclassified IBD (IBDU) (156, 208). A study by Kolho et al. (191) investigating faecal and serum metabolomics in paediatric IBD patients included subjects with IBDU but then combined this group with UC patients in the analysis as the IBDU cohort was too small.

1.2.3.1 IBD metabonomic profiling of urine

Metabonomic profiling in IBD using urine samples has been explored quite extensively in human studies. Urine is an easily attainable biofluid, and requires little preparation for NMR analysis compared to other biological samples. It gives time-averaged (between micturition) information of the endpoints of both endogenous and exogenous (medication and diet) metabolism, and contains the gut bacterial metabolites and host-bacterial co-metabolites (156). Table 1.2 summarises the studies published prior to this work.

Study	Samples / analytical platform	Changes in metabolites in IBD cohorts compared to controls	
		Untargeted analysis	Targeted analysis
Dawiskiba 2014 (209)	CD (n =19) UC (n =24) HC (n=17) ^{1}H NMR	↓ hippurate ↓ trigonelline ↓ taurine ↓ succinate ↓ 2-hydroxyisobutyrate	↓ hippurate ↓ citrate ↓ alanine ↓ formate ↓ trigonelline ↓ taurine ↓ succinate ↓ glycine ↑ 2-hydroxyisobutyrate ↑ acetoacetate
Stephens 2013 (210)	CD (n = 30) UC (n = 30) HC (n = 60) ^{1}H NMR	↓ hippurate ↓ formate ↓ methanol ↓ acetate ↓ methylamine ↓ succinate ↓ trans-aconitate ↓ citrate ↓ 1-methylhistidine ↓ histidine ↓ lysine ↓ asparagine ↓ trigonelline ↓ creatine ↓ taurine	not done
Schicho 2012 (207)	CD (n = 20) UC (n = 20) HC (n = 40) ^{1}H NMR	↓ hippurate ↓ citrate ↓ succinate ↓ 1-methylnicotinamide ↓ trigonelline ↓ betaine ↓ formate ↓ 1-methylhistidine ↑ acetate	not done
Bjerrum 2010 (UC only) (211)	UC (n = 68) HC (n = 25) ^{1}H NMR	no difference	not done
Williams 2009 (161)	CD (n = 86) UC (n = 60) HC (n = 60) ^{1}H NMR	↓ hippurate ↓ citrate ↓ NNN-trimethyllysine ↑ guanidoacetate, ↑ glycine ↑ methylhistidine ↑ glycolate	↓ hippurate ↑ formate ↓ 4-cresol sulfate

Table 1.2: Table of metabonomic studies using urine sampling – adapted from (212) and (213)

The first study to show differences between urine metabolic profiles between IBD and controls was published by Williams et al (161) in 2009, and was also able to separate Crohn's disease (CD) from ulcerative colitis (UC) with discriminatory metabolites that were principally related to intestinal microbiota. However, only Caucasian patients were recruited, and those with significant other comorbidities were excluded. Ten of the 86 Crohn's patient and 8 of the 60 UC patients had evidence of a disease flare, with an HBI score of ≥5 or a SCCAI score ≥5 respectively. This study used ^{1}H NMR spectroscopy, and initial separation between IBD and control cohorts using untargeted multivariate analysis was driven by the presence of xenometabolites, and specifically metabolites attributable to paracetamol and 5-aminosalicylates. Consequently, patients identified as taking these medications from the corresponding metadata were excluded from further analysis. The subsequent loadings plot showed that separation was then driven principally by hippurate, a product of the microbial metabolism whose synthesis is related to the presence of *Clostridia* species, known to be lowered in IBD. The main discriminatory metabolites for Crohn's disease when compared to controls using OPLS-DA analysis were hippurate, guanidoacetate, glycine, methylhistidine, citrate, and glycolate. UC differentiated from controls with changes in relative quantity of methylhistidine, guanidoacetate, hippurate, citrate and glycine. Between CD and UC, changes in citrate, hippurate, methylhistidine, guanidoacetate and 4-cresol sulfate were discriminatory.

Hypothesis-led targeted analysis showed lower levels of hippurate between IBD and controls, and increased levels of formate, with the latter's production being related to the *Enterobacteriaceae* family of bacteria, particularly Escherichia coli. 4-cresol sulfate was also shown to be reduced in the IBD cohort on targeted analysis, with its production again related to the presence of *Clostridia* species. Concentration of these 3 metabolites were different between the Crohn's disease and UC cohorts too, with lower excretion of hippurate and 4-cresol sulfate, and increased excretion of formate in the Crohn's disease cohorts. Hippurate was also

significantly lower in isolated colonic Crohn's disease compared to all UC, and UC pancolitis. Effect of disease activity, measured using HBI and SCCAI scores, on urinary metabolic profiles was assessed in this study, but no significant differences were seen discriminating active and quiescent disease on targeted analysis.

The study authors detail how they addressed potential confounders; they excluded patients with significant comorbidities, intercurrent illnesses, biologics, and those on treatment diets (EEN etc), antibiotics and pre- and probiotics. Patients who were pregnant, and those with stomas were excluded. Detailed information including diet (including 24 hours recall of foods and substances known to affect the urinary metabolome), medications, female menstrual information, exercise, alcohol, and smoking status were taken and analysed between cohorts. Effect of body mass index (BMI) was not reported (161).

A study by Schicho et al (207) collected serum, plasma and urine from 20 subjects with Crohn's disease, 20 with UC subjects, and 40 healthy controls and performed ^{1}H NMR spectroscopy on each biofluid. All patients were fasted at the time of collection. Patients with significant comorbidities or an inter-current illness, or those on biological treatment, antibiotics or pre- or probiotics were excluded. Resonances corresponding to metabolites related to medication, including paracetamol and acetamide, were removed from the spectral analysis. 3 Crohn's disease and 15 UC patients were taking 5-ASAs at the time of analysis, and it is not clear from the study report how the related metabolites, which are known to interrupt the aromatic region of the urinary ^{1}H NMR spectrum (161), were accounted for. Using untargeted OPLS-DA modelling of urine samples, Crohn's and UC could be separated from controls, with stronger separation in the Crohn's disease cohort. 26 metabolites were changed in Crohn's disease compared to controls, whereas only 23 were altered in UC. There were only a few metabolites that were altered in both, including hippurate, citrate, succinate, betaine, and

methanol. Citrate and succinate are TCA cycle intermediates, and betaine (also known as trimethylglycine) is mainly acquired from the diet, and there is growing evidence that it has an anti-inflammatory effect by inhibiting nuclear factor-κB (214). Separation could not be established between Crohn's disease and UC using supervised multivariate analysis. TCA cycle intermediates, including citrate and succinate, were lower in UC compared to the control cohort. This study did not report disease activity in the patient information section of study, and did not assess effect of disease activity on the metabolome.

This study reported lifestyle information as detailed above, and assessed for any significant impact between comparison groups. Fasting samples were taken to help prevent diet potentially influencing the metabolome, but otherwise no specific diets were excluded. Subjects with comorbidities were excluded, and mean height and weight (and ranges) between Crohn's disease, UC and control groups were assessed and reported as similar between cohorts, but BMI comparisons were not stated (207).

Untargeted analysis of urine samples was performed by Stephens et al (210), where 30 Crohn's disease patients, 30 UC, and 60 controls were recruited. Four of the Crohn's patients and 19 of the UC patients were taking 5-aminosalicylate based treatment, but it is unclear how these were accounted for in the paper manuscript. Patients were excluded if they had urinary dysfunction or infection at the time of recruitment. Other comorbidities, BMI and other potential confounders were not reported. Harvey Bradshaw Index (HBI) was used to assess disease activity in Crohn's disease participants, and the Mayo Disease Activity Score (MDAS) was used in UC subjects. 13 patients in the Crohn's cohort and 17 in the UC cohort were regarded as being in clinical remission. Multivariate analysis using OPLS-DA was able to distinguish IBD from control cohorts. Discriminatory metabolites included ones derived from gut microbiota including hippurate, formate, acetate, methylamine and methanol. TCA cycle

associated metabolites including succinate and citrate, and several amino acids including histidine were also discriminatory in separation between IBD and controls. Crohn’s disease patients could only be distinguished from UC if the model included patients who had undergone intestinal resection. IBD disease activity information was collected using Harvey–Bradshaw Index and Mayo Disease Activity Score for Crohn's disease and UC respectively. However, effect of disease activity on the metabolome was not reported in this study.

A study by Dawiskiba et al (209) assessed urinary metabolic profiles in 19 Crohn’s disease patients, 24 UC patients, and 17 healthy controls. Samples were taken following an overnight fast, and patients were excluded if they had an infection, and any other ‘severe diseases’ including diabetes mellitus, liver or kidney impairment, and malignancy. No information about BMI was described in this paper. Signals in the NMR spectrum related medications including 5-aminosalicylate, azathioprine and acetaminophen were removed, and so not included in the analysis. This study showed the strongest separation using partial least squares-discriminative analysis (PLS-DA) was between active IBD and control cohorts, and between IBD active and remission cohorts. In the former, hippurate, citrate, succinate, trigonelline, taurine, and 2-hydroxyisobutyrate were the main discriminatory metabolites. In both active and remission cohorts, hippurate, citrate, succinate, and taurine were discriminatory between IBD and control cohorts. Higher concentrations of glycine and lower concentrations of acetoacetate separated active IBD from disease remission. Targeted univariate analysis was subsequently preformed on metabolites identified as driving the PLS-DA models, with discriminatory metabolites listed in Table 1.2. Mean IBD activity score (SCCAI/HBI) data was presented in a patient clinical profile table, but no assessment of activity on the metabolome was reported in this study.

Bjerrum et al. (211) used NMR spectroscopy and multivariate statistics to assess for potential biomarkers in ulcerative colitis only, and showed no separation between the UC cohort and

control based on urinary metabolic profiling. In this study, urine samples were collected from 41 active UC patients, 33 quiescent UC patients, and 25 controls. Several potential confounders were accounted for including active infections, recent use of antibiotics, severe mental illness, and pregnancy. It did not report whether patients with other significant diseases such as diabetes mellitus, cardiovascular or respiratory disease were excluded. Participant BMI was not stated in the main manuscript. The NMR spectra had regions removed with no clear reason given (it is assumed that this was done to exclude drug metabolites including those for 5-ASAs), and this included the aromatic region where most of the discriminatory metabolites for IBD have been found in previous studies (215). Disease activity was assessed on colonic tissue samples, but in the urinary NMR results the effects were not reported, but this is likely because UC could not be differentiated from controls.

Overall, it is difficult to comment on the homogeneity of populations examined in the above studies as although participant characteristics were described in varying details in the above five studies, only exclusion of subjects with other significant diagnoses was reported in three of these studies. Obesity, another important health condition, is only addressed in one of the five studies above, where mean height and weight were compared between disease and control cohorts, although no BMI or statistical analysis was described.

1.2.3.2 IBD metabonomic profiling of faecal water

With the recent interest of the intestinal microbiome in the pathogenesis of IBD, faecal sampling has been seen as an ideal biological sample type to examine the corresponding metabolic effect, and consequently there have been several metabonomic studies using faecal extracts to examine for discriminatory metabolites in IBD. Faecal water or extracts have the advantage that they contain direct metabolic information of the microbiome within the environment where it is located, and samples are non-invasive to obtain. Disadvantages include

patients often cannot provide a sample on demand, and so often have to return to hospital, the acceptability from patients of providing and handling samples, and these samples often require more preparation than urine. Faecal samples will also be heavily influenced by potential confounders including xenobiotics (molecules not naturally found within an organism) include metabolites related to drugs and nutrition. The following table summarises the faecal metabonomic studies in IBD to date.

Study	Samples	Analytical platform	Changes in metabolites in IBD cohorts compared to controls
Santoru 2017 (216)	CD (n = 50) UC (n = 82) HC (n = 51)	^{1}H-NMR GC-MS LC-MS	↑ biogenic amines ↑ amino acids, ↑lipids ↓ B group vitamins
Bjerrum 2015 (217)	UC (n = 48) CD (n = 44) HC (n = 21)	^{1}H NMR	↑ amino acids ↓ SCFAs in IBD
Le Gall 2011 (218)	UC (n = 13) HC (n = 22)	^{1}H NMR	↑ taurine ↑ cadavarine ↓ acetate ↓ butyrate
Jansson 2009 (219)	CD (n = 10 twin pairs, 6 are discordant) HC (n = 7 twin pairs)	ICR-FT/MS	↑ amino acids ↑ bile acid metabolites
Marchesi 2007 (220)	CD (n = 10) UC (n = 10) HC (n = 13)	^{1}H NMR	↓ acetate ↓ butyrate ↓ methylamine ↓ trimethylamine

Table 1.3: Table of metabonomic studies using faecal extracts in IBD – adapted from (212) and (213)

The first study published assessing the impact of IBD on faecal metabolites was by Marchesi et al. (220), where ^{1}H NMR spectroscopy was performed on 10 Crohn's disease patients, 10 UC

patients, and 13 controls. IBD patients were being treated with prednisolone and 5-aminosalicylates as part of their clinical management. Information regarding comorbidities, other medications or BMI was not reported. Initial analysis showed that the presence of metabolites related to 5-aminosalicylates were driving separation in multivariate analysis, and so the entire aromatic region was subsequently removed from the spectrum prior to further analysis. Depletion of particularly acetate and butyrate, short chain fatty acids (SCFA), was the main factor driving separation between Crohn's disease patients and healthy controls. These metabolites are produced via fermentation of complex carbohydrates by intestinal bacteria (221). Methylamine and trimethylamine were also decreased, with these metabolites being derived from degradation action of the microbiota on the choline and carnitine. These findings correlate with microbial studies in IBD that have shown a reduction in *Clostridia* species *C. leptum* and *C. coccoides* that are principally responsible for producing SFCAs in the gut. This study also showed a reduction in butyrate, methylamine and trimethylamine in UC. Effects of disease activity on the faecal metabolome was not described in the study manuscript.

Jansson et al (219) used ion cyclotron resonance fourier transform mass spectrometry (ICR-FT/MS) to perform non-targeted metabolic profiling to assess for differences in gut microbiota associated metabolites in faecal samples between Crohn's disease patients and controls. ICR-FT/MS is a very sensitive technique able to differentiate between subtle variations between thousands of metabolites including those with higher molecular weights. Consequently, this study recruited identical twins to help identify metabolites specific to Crohn's disease within the thousands that could be detected using this technique. 17 pairs of identical twins were recruited, with 7 healthy sets, 4 who were discordant for predominantly colonic CD, 2 discordant for predominantly ileal CD, 2 concordant for ileal disease, and 2 concordant for colonic disease. All disease participants had inactive disease based on HBI score. Metadata regarding comorbidities, BMI and medication was not reported in the paper. PL-DA

multivariate analysis showed differing concentrations of bile acid metabolites including glycocholate and taurocholate, metabolites related to fatty acid biosynthesis including oleic and stearic acid, and amino acids tyrosine and tryptophan.

^{1}H NMR spectroscopy of faecal water samples was used by Le Gall et al (218) to investigate the changes in faecal metabolic profiles between UC, irritable bowel syndrome (IBD) and control subjects. 13 UC patients, 10 IBS, and 22 controls were recruited. All but one of the UC subjects were taking 5-aminosalycilate treatment, and spectral regions containing 5-ASA metabolites including the entire phenolic region were removed from the analysis. Details regarding BMI, medications and other comorbidities were not reported. Multivariate discriminant analysis using PLS-DA modelling was able to differentiate UC from controls, reporting a slight reduction in acetate and butyrate, and an increase in taurine and cadaverine. Disease activity was assessed in this study by measuring faecal calprotectin (FCP), and those with a FCP level less than 75 mg/kg (n = 6) were compared with subjects with a FCP greater than 125 mg/kg (n = 7), and an elevated faecal lactate was detected in the higher FCP group.

A larger study by Bjerrum et al. (217) recruited 48 patients with UC, 44 with Crohn’s disease, and 21 controls, and analysed faecal extracts using ^{1}H NMR spectroscopy. Crohn’s and UC cohorts were split into groups of active and inactive disease using HBI and Mayo scoring systems respectively, and these groups were compared to controls and each other. Detailed IBD clinic information was reported including disease activity and IBD medications. Body mass index and the presence of other comorbidities were not reported. 42 of the UC cohort and 2 of the Crohn’s cohort were taking 5-aminosalicylate based treatment, and regions in the spectra where 5-ASA metabolites were present were subsequently removed. Faecal metabolic profiles were able to differentiate active and inactive IBD from controls, and Crohn’s from UC, although the discriminatory power of the latter was lost when patients who were on anti-TNF treatment or those who had previous bowel resection were eliminated from the cohort. Active

UC could be differentiated from inactive UC, but this was not replicated for Crohn's disease. As with previous studies, discriminatory metabolites were microbiota-related SCFAs including butyrate, and several amino acids including alanine, tyrosine, and phenylalamine.

A study by Santuro et al. (216) took more of an 'inter-omics' approach, analysing both faecal metataxanomic and metabolic data. 50 Crohn's disease, 82 UC and 50 healthy controls were recruited and faecal samples were analysed using ^{1}H NMR spectroscopy and 16S rRNA sequencing. Participant clinical information including the presence of comorbidities and BMI was not reported in the main paper report. OPLS-DA score plots separated IBD patients from controls. 14 metabolites were discriminatory in the Crohn's disease cohort, including an increase in two biogenic amines, putrescine and cadaverine, produced by intestinal bacteria, and were shown in this study to negatively correlate with the amount of two bacterial genera *Faecalibacterium* and *Oscillospira*, which belong to the *Firmicutes* phylum. Nicotinic acid and pantothenic acid, two group B vitamins, were also decreased in the Crohn's cohort, and the former was directly correlated to the decreased abundance of *Faecalibacterium prausnitzii* found. Methylamine was decreased in the Crohn's cohort, and is derived from intestinal degradation from dietary compounds including carnitine and choline, and its depletion correlated with a decrease of *Oscillospira*.

1.2.4 Potential confounders that may affect metabonomic analysis in IBD

Studies to date assessing urinary and faecal metabolic profiles in IBD have either excluded patients with other comorbidities because of potential confounding (205, 209, 215), or have not stated whether patients with comorbidities were recruited (211, 216-220). These studies have also not accounted for the possible confounding effects of obesity, or the use of bowel cleansing for endoscopic procedures that these patients frequently undergo. For urinary metabonomics to be applied to a real world IBD population, the changes seen in these IBD

urinary metabolic profiles must be applicable to the whole population, including obese patients, those with other comorbidities, and those who may have undertaken recent colonoscopy. Several common conditions that have been shown to affect either the urinary or faecal metabolome including obesity and the use bowel cleansing are discussed below.

1.2.4.1 Diabetes mellitus

Most studies investigating metabolic changes related to diabetes (207, 210, 211) mellitus have assessed plasma or serum (222, 223). However, both type 1 and type 2 diabetes mellitus have been reported to influence the urinary metabolome (224). Messana et al (225) compared urinary metabolites of patients with type 2 diabetes mellitus and controls, and demonstrated higher levels of the bacterial associated metabolites hippurate, trimethylamine N-oxide, and dimethylamine in the diabetic cohort. Zhao et al (226) used untargeted metabonomics to assess pre-diabetic traits in urine and plasma, and showed gut flora associated metabolites were discriminatory in patients with impaired glucose tolerance (IGT), including changes in IBD associated hippurate. Higher excretion of hippurate has also been observed in a study assessing urinary metabolic changes in type 1 diabetes mellitus relative to healthy controls (227).

1.2.4.2 Asthma

Metabolic profiling has been applied to asthma to help investigate the relationship between pathophysiologic characteristics and particular clinical features or treatment response using a range of biological samples including serum/plasma, urine, breath, and bronchiolar lavage (228). A study by Saude et al. (229) was able to separate asthmatic patients from controls based on different levels of urine metabolites using ^{1}H NMR spectroscopy. These metabolites included intestinal bacterial associated metabolites hippurate and trimethylamine N-oxide (TMAO). Another study by Quan-Jun et al (230) demonstrated changes in urinary TMAO in

patients with asthma exacerbations being treated with combination inhaled salbutamol and budesonide.

1.2.4.3 Effect of other diseases

Urine contains both host and microbial products, and so urinary metabonomics has been applied to a wide range of conditions that are influenced by both. As urine contains metabolites produced by intestinal bacteria or host-bacteria interactions, it has been used to study several dysbiosis associated GI conditions including IBS, antibiotic associated diarrhoea, as well as IBD. It has also been applied to other microbiota associated conditions such as tuberculosis, autism, and metabolic syndrome (231).

Metabolic profiling has been used extensively in cardiovascular research, although principally with serum/plasma (232, 233). Urinary metabolic profiling has been studied in hypertension, which has shown changes in bacterial associated metabolites including hippurate and dimethylamine (234). Acute heart failure with renal impairment has been shown to alter the composition of urinary metabolites (235), and patients with cerebral vascular disease have lower levels of metabolites including hippurate in their urine (236). Hippurate and formate, the latter another intestinal associated urinary metabolite, have been shown to be influenced by lung function in patients with COPD (181).

Rheumatoid arthritis, along with other inflammatory conditions, have been reported to show differentiation from healthy controls in urinary metabonomic experiments (237, 238). Changes in urinary metabolites have also been observed to distinguish cancer patients from controls (239), and in a study by Carrola et al (240), the relative quantity of the bacterial associated metabolite hippurate was found to be lower in patients with lung cancer.

As faecal samples directly contain the metabolites of intestinal bacteria and bacteria-host co-metabolism, they have been widely used as a research tool for gut microbial associated conditions (241). As well as GI disorders including, IBD, IBS, antibiotic associated diarrhoea (AAD), *Clostridium difficile* infection, and colorectal carcinoma, other non-GI microbial associated conditions have been investigated. These include obesity (described above), and liver cirrhosis. In liver cirrhosis, non-targeted reversed-phase ultra-performance liquid chromatography coupled to electrospray ionization quadrupole time-of-flight mass spectrometry (Q-TOF-MS) has reported a reduction in bile acids, and an enrichment of aromatic amino acids and fatty acids (242).

1.2.4.4 Obesity and IBD

The prevalence of IBD patients with obesity is similar to the general population (243, 244), and this is in the context of a dramatic rise in obesity worldwide (245). Several studies have assessed the clinical impact of obesity in patients with IBD, and have reported that a high body mass index (BMI) may influence the nature of this condition, and the time of onset to developing peri-anal disease and first surgery, along with disease-related quality of life, and CRP levels. (17, 20, 21, 246, 247). However, other studies have shown a more benign disease course associated with adiposity (19, 22). Obesity has also been reported as influencing disease location, with isolated colonic Crohn’s disease being more prevalent in patients with a higher BMI (247).

Two observational studies using large patient cohorts have shown a higher risk of developing Crohn's disease in obese subjects, with the United States Nurses' Health Study II (248) of over 110,000 women showed a greater two fold risk of developing this disease at the age of 18 years old, and the Danish National Birth Cohort (249) of over 75,000 women showed a nearly 2 fold risk increased risk.

Obesity refers to excessive body adipose tissue, and the diagnosis is normally made by measuring a person's body mass index (BMI), calculated by the weight in kilograms divided by the square of height in meters, with a value of greater than 30 kg/m^2. However, BMI is not a direct measure of adiposity, as it does not differentiate excess fatty tissue and lean body mass. Measuring waist circumference has a better association with degree of visceral adipose deposition compared to BMI (250). There are several methods for calculating body fat percentage, although the best direct method is with using dual-energy X-ray absorptiometry (DEXA). Body fat percentage correlates better than BMI with metabolic syndrome and type 2 diabetes (251), and waist circumference correlates better than BMI with cardiovascular risk (252). High BMI can also mask sarcopenia, defined as a loss of skeletal muscle mass, and as some individuals have sarcopenic obesity, where they are both obese and sarcopenic (253). Sarcopenic obesity typically affects people over the age of 60 years, and has its own impact on physical capacity and health (253). Studies assessing the effect of obesity on inflammatory bowel disease have used BMI as a measure of obesity (17, 20, 21, 243, 246, 247), and this may be in part the reason for the difference reported effects of obesity in IBD patients.

There are several plausible mechanisms for how obesity may affect IBD. Up to half the body mass of morbidly obese individuals can comprise of adipose tissue, distributed mainly intra-abdominally and subcutaneously (254). Adipose tissue is highly physiologically active, with visceral adipose tissue having a significant role in promoting local and systemic inflammation (255). Adipose tissue contains immune cells including T and B lymphocytes, and visceral adipose tissue contains more pro-inflammatory M1 macrophages which secrete pro-inflammatory cytokines involved in the pathophysiology of IBD including TNF-α and IL-1 (256, 257).

Fat-wrapping, or creeping fat, has also been associated with Crohn's disease, with Crohn et al. (258) first reporting the phenomena as the presence of enlarged mesenteric adipose tissue enveloping the corresponding inflamed intestines. The adipocytes within creeping fat are distinct from normal adipocytes as they are smaller with higher section of adipokines. Adipokines, also called adipocytokines, are cytokines produced by adipocytes.

There is increased bacterial antigen translocation which occurs through the transmural inflammation in Crohn’s disease, and are detected by innate adipocyte receptors, which in turn mediate the local inflammatory response (25). Visceral adiposity is modulated by growth hormones that are reduced in Crohn's disease (259). However, there has been no clear correlation shown between an increase in visceral adiposity and BMI in Crohn's disease (260).

Adipocytes and pre-adipocytes produce over 50 adipokines, including IL-6 and TNF-α. There is more expression of TNF-α and IL-6 in IBD, with the amount of secretion correlating with mesenteric adipocyte load (255, 257). There is also increased expression of other adipocyte secreted inflammatory mediators such as the hormone leptin, with over-expression in mesenteric adipocytes and in the colonic lumen of IBD patients (261, 262). Adiponectin and resistin are other pro-inflammatory mediators produced by adipocytes that may have a role in IBD due to their effects on toll like receptor via the nuclear factor-kB pathway (263) and expression on pro-inflammatory cytokines (264) respectively.

Obesity related metabonomic research has focused on using plasma/serum (265), but several animal and human studies have been conducted using urine (266-268). Kim et al. (268) showed that levels of tricarboxylic acid cycle (TCA) related metabolites and the gut bacteria associated metabolite phenylacetylglutamine differed in the obese rat model induced by high-fat diet compared to normal diet rats. A large study (267) that used the samples of over a thousand human subjects analysed the correlation of body mass index with urinary metabolic profiles,

and identified nine intestinal bacterial associated metabolites which were affected by increasing body weight. These gut microbial metabolites include trimethylamine, dimethylamine, 4-cresol sulfate, phenylacetylglutamine and 2-hydroxyisobutyrate. A study by Schwiertz et al. (269) assessing the effects of obesity on faecal SCFAs showed that overweight and obese groups had higher levels of butyrate, propionate, acetate compared to controls. Hippurate has been observed to have a significant inverse relationship with adiposity (267, 270).

The impact of BMI on metabolic profiles of other conditions has been assessed in type 2 diabetes, where BMI had no confounding effect on type 2 diabetes mellitus related metabolites using meta-regression in a large systemic review and integrative analysis (271). A study investigating the relationship between clinical characteristics and asthma associated urinary metabolites excluded patients with a BMI ≥ 30 kg/m^2 (272). The influence of BMI on rheumatic disease assessed BMI as a confounder, and showed no effect with BMI on elevated branched-chain amino acids associated with osteoarthritis (237). Several studies assessing metabonomics in cardiovascular disease, asthma, and lung cancer have not assessed BMI as a potential confounding factor (230, 232, 240, 273).

1.2.4.5 Bowel purgatives for colonoscopy

Colonoscopy is performed to assess gastro-intestinal symptoms, and is used frequently in the screening and surveillance of IBD patients and patients with colonic polyps. This procedure requires the use of bowel cleansing agents for safe intubation and good visualisation of the large intestine. Polyethylene glycol (PEG) solutions are the most commonly used and preferred bowel cleansing agent for bowel preparation prior to colonoscopy (274, 275). PEG causes a profound osmotic diarrhoea with a high-volume lavage rapidly passing through the gastro-intestinal tract, which in turn alters the luminal contents including the microbiota and luminal metabolites (276, 277).

It has been increasingly recognised over the last few years that, along with use of antibiotics (90), studies of the microbiota need to take in to account the potential confounding effects of bowel preparation using bowel cleansing agents, as several studies have shown bowel preparation to temporarily affect both the faecal and the mucosal associated microbiota (276-280).

Compositional studies assessing changes in the faecal associated microbiota following bowel preparation have shown significant effects on bacterial load, overall bacterial diversity (α-diversity), and composition (β-diversity) (276, 277, 280, 281). A study by Jalanka et al. (281) showed bacterial load was significantly reduced by up to 35 fold in samples immediately post lavage after high volume preparation with polyethylene glycol (PEG), and microbiota composition changed to the extent that subject-wise clustering was lost, although these changes recovered within 14 days. Two studies (276, 280) shown that bowel cleansing reduces α-diversity, which is a measure of compositional diversity of bacteria by assessing richness and evenness of the bacterial taxa. Richness, that reflects the number of different bacteria in a sample (280), was reduced in both studies (276, 280) following induction of an osmotic diarrhoea with PEG. Evenness, that reflects how uniformly distributed the different taxa are within samples, was not affected (276, 280). Shobar et al. (280) also showed a trend towards a reduction in the Shannon index, a measure that takes into account both richness and evenness. Three other studies have shown no effect of bowel preparation on faecal α-diversity (277, 278, 282). Differences in β-diversity, which reflects compositional changes between samples (280), have also shown changes in the faecal associated microbiota following bowel preparation (277, 280).

Taxanomic composition analysis of the faecal associated microbiota has shown significant changes between pre- and post-bowel preparation samples in four studies (276, 278, 280, 281).

However, these findings have not been consistent between studies. At phyla level, the relative quantity of Firmicutes and Bacteroidetes have varied in different studies (276, 278, 280), and two studies have shown an increase in Proteobacteria (278, 281). Changes at different taxa levels down to genus level have be reported, but these are also conflicting between studies (276, 278, 280, 281). Three studies have shown no significant taxanomic compositional changes at phyla level (277, 282, 283). These conflicting results are likely due in part to small subject numbers, and differing study design and analysis.

One study to date has examined the effect of bowel purgatives on the metabolome. Nagata et al. used Spearman's correlation coefficients to examine differences in faecal metabolites at baseline, day 1, and day 14 post bowel cleansing in eight patients scheduled to undergo colonoscopy. Unsupervised multivariate analysis using principal coordinate analysis (PCoA) showed non-clustering of samples belonging to the same patient immediately post bowel cleansing (day 1) in 6 of the 8 patients, with the remaining time points being clustered in these patients. Targeted analysis was performed using capillary electrophoresis time-of-flight mass spectrometry (CE-TOF-MS) to assess changes in 514 faecal metabolites. 32 metabolites, including amino acids and short chained fatty acids (SCFAs) significantly changed following bowel cleansing, but then recovered at 14 days.

1.2.5 Novel techniques in faecal metabolic profiling

IBD studies to date have principally used ^{1}H NMR spectroscopy (161, 207, 209-211, 216-218, 220) as the analytical platform to assess urine and faecal metabolic profiles, although mass spectrometry has also been used in two studies assessing faecal extracts (216, 219). ^{1}H NMR spectroscopy has the disadvantage that it can lack sensitivity, particularly in the presence of multiple potential confounders. Mass spectrometry is a more sensitive platform, and but involves extensive sample preparation and destruction of samples during the analytical process.

Rapid evaporative ionisation mass spectrometry (REIMS) is a relatively new technique that has not been applied to IBD metabolic profiling, and involves rapid analysis of samples requiring minimal preparation to obtain lipidomic spectral profiles and has a greater sensitivity than NMR spectroscopy. This technique has not been explored in this thesis.

1.3 Scope of this thesis

IBD metabonomic research has been restricted to homogeneous clinical cohorts where subjects with comorbidities have been excluded. Other factors present in a real-life IBD population that may influence the metabolome include obesity and the effects of bowel cleansing pre-colonoscopy. This thesis aims to assess these specific factors on the IBD metabolome, examining principally urine as this biofluid is easily collected and processed, and gives a wealth of metabolic information including the endpoints of intestinal microbial metabolism.

Chapter 2: Materials and Methods

There is a separate materials and methods chapter in this thesis to prevent repetition as the three studies use the same techniques. Aspects of the materials and methods specific to individual studies will be further described within the individual chapters.

Chapter 3: Examining the differences in urinary metabonomic profiles of a real-life IBD population and relating it to changes in the faecal microbiome.

The first part of this chapter examines the faecal microbiomic composition between IBD and healthy control subjects to assess whether differences can be linked with changes seen in the urinary metabolome.

The second part of this chapter examines whether differences in the urinary metabolome associated with inflammatory bowel disease can be seen in a real-life population of subjects, including those comorbidities that have been excluded from previous studies.

Hypothesis: Urinary metabolic profiles associated with IBD are specific to this condition and will therefore present when applied to a cohort of subjects representing a real-life clinic population, and this will include subjects with comorbidities.

Chapter 4: Effects of obesity on the IBD urinary metabolome.

This chapter examines the effects of obesity on the urinary metabolome of IBD subjects, and specifically its effect on urinary metabolites known to be discriminatory in IBD.

Hypothesis: Discriminatory metabolites in IBD are specific to this disease and will not be influenced by high body mass index (BMI).

Chapter 5: Effects of bowel purgatives on urinary and faecal metabolic profiling.

This study aimed to compare the effects of bowel cleansing on the faecal microbiome and urinary and faecal metabolome at baseline, day 3 and week 6 after colonoscopy.

Hypothesis: Bowel cleansing using polyethylene glycol will have a transient and temporary effect on urinary and faecal metabolome correlating with a temporary change in the faecal microbiome.

Chapter 2: Materials and Methods

2.1 Subjects and clinical data

All studies had ethical approval from Imperial College Healthcare NHS Trust Research and Ethics Committee (Ref: 13/LO/1867). IBD patients were recruited from gastroenterology clinics at St Mary's Hospital in Paddington, and Ealing Hospital in Southall. Patients were collected for this thesis by myself, Dr Lucy Hicks, and Dr Leo Chong, for our concurrent studies – all recruitment and collection of samples were done using the same protocols and under the same ethical approval.

Verbal and written information was given to all patients, and informed written consent was acquired from all study participants.

2.1.1 Recruitment of IBD patients

Clinical information was acquired from patients directly, and use of their medical notes and investigation reports. IBD patients were only recruited if there was a clear diagnosis of either Crohn's disease or ulcerative colitis based on clinical information, and radiological, endoscopic and histological test results. Patients with IBD unclassified were excluded. Patients were sub-classified using the Montreal classification system (284), and disease activity at the time of recruitment was recorded using the Harvey-Bradshaw index (HBI) (285) or the simple clinical colitis assessment index (SCCAI) (286) scoring systems for Crohn's disease and ulcerative colitis respectively. A disease activity score of greater than 5 in both scoring systems was considered active disease.

Details of diagnosis, investigations, past and current IBD medication, and previous operations was all recorded. IBD medications were classified as 5-aminosalicylates, immunosuppressive

(thiopurines, Tacrolimus, Methotrexate, Mycophenolate), and biologic (Infliximab, Adalimumab or other biologics).

2.1.2 Comorbidity and obesity control cohorts

IBD patients with obesity and comorbidities were recruited from gastroenterology clinics at St Mary's Hospital and Ealing Hospital. Control patients with comorbidities were recruited from metabolic, endocrinology and respiratory clinics at St Mary's Hospital. Patients were recruited to match comorbidities in the IBD cohorts, and patients with co-existing gastroenterological disease other than IBD, such as irritable bowel disorder, were excluded from the control cohort. Details of other medications including prescribed, over-the-counter and herbal medications, were also recorded. Patients taking probiotics, prebiotics, or antibiotics within 2 months of the time of recruitment were excluded.

2.1.3 Metabonomic information

Along with age and sex of the patient, further information was collected that may influence metabolic profiling. This included body mass index (BMI), details about menstruation, smoking, alcohol intake, exercise and 24-hour recall on diet. Details regarding specific dietary components (such as fish, liquorish, cherries, tea, coffee and herbal tea) that are known to affect the NMR spectrum (161) was also obtained.

2.2 Sample collection and preparation

2.2.1 Urine samples

Random urine samples, as opposed to first void, were collected from study participants as they are subject to less intra-individual variability influenced by lifestyle and diet (287). Samples were collected in 30ml universal containers (Sigma-Aldrich, USA).

Within 6 hours of production, samples were centrifuged for 10 minutes at 13,000 rpm at 4 ºC, and the supernatant was then aliquoted off and transferred into a microcentrifuge tubes (Ependorff, Germany) and stored at -80 ºC until used for NMR analysis.

2.2.2 Faecal sample collection and water extraction for NMR

Faecal samples were collected using spoon cap sample containers (Sigma-Aldrich, USA), and were then separated into microcentrifuge tubes (Ependorff, Germany) prior to being frozen at -80 ºC on the same day (within 12 hours of being produced).

Prior to NMR analysis, 500 mg of crude stool was mixed with two volumes of phosphate buffer saline (PBS) solution, and then transferred into vortex conical tubes before being vortexed at 2000 Hz for 15 minutes. These were then centrifuged at 9500 rpm for 20 minutes at 4 ºC, and 600 µl of supernatant was then aliquoted into microcentrifuge tubes before NMR analysis.

2.2.3 Faecal DNA extraction for microbial analysis

Faecal DNA extraction was performed using PowerLyzer PowerSoil DNA Isolation Kit (Mo Bio, Carlsbad, CA, USA). This protocol homogenizes the faecal samples using a bead beating tube, followed by mechanical and chemical lysis of the bacterial cells, allowing total genomic DNA capture on a silica membrane in a spin column format, where the bacterial DNA is then washed and eluted from the membrane ready for PCR analysis.

For each sample, 250 mg of crude stool was added to the PowerBead Tube provided, and votexed for 30 seconds. 60 µl of Solution C1 was then added and the mixture was votexed for 10 minutes. The tube was centrifuged at 10,000 x g for 30 seconds, and then 250 µl of Solution C2 was then added. This mixture was centrifuge for 1 minute at 10,000 x g, and 600 µl of the resulting supernatant was transferred to a clean collection tube. 200 µl of Solution C3 was then

added to the collection tube, and the mixture was incubated for 5 minutes at 4 ºC. The tube was centrifuged at 10,000 x g for 1 minute, and 750 µl of the supernatant was then transferred to clean collection tube. 1200 µl of Solution C4 was added to the collection tube, and the mixture was then vortexed for 5 seconds. 675 µl of the mixture was then loaded onto a spin filter and centrifuged for 1 minute at 10,000 x g, with the flow through then discarded. A further 675 µl of supernatant underwent the same step, followed by the remaining supernatant that was left, with each time the flow through was discarded. 500 µl of Solution C5 was then added to the spin filter, and then was centrifuged at 10000 x g for 30 seconds. The flow through was discarded and the spin filter was centrifuged for 1 minute at 10,000 x g. The spin filter was then placed in a 2ml clean collection tube and then 100 µl of Solution C6 was added to the white filter membrane and centrifuged for 30 seconds at 10,000 x g. The spin filter was then discarded and the remaining fluid in the collection tube contained DNA which was frozen at -20 ºC prior to analysis.

Prior to 16S rRNA sequencing analysis, a QubitTM fluorometer and quantitation assays were used to quantify DNA in each sample. A fluorometer was calibrated using standards #1 and #2 prior to 200 µl of each sample being transferred to 0.5ml Qubit® assay tubes. Samples were incubated for 2 minutes at 20 ºC prior to being read on fluorometer providing DNA concentrations in ng/mL.

2.3 16S rRNA sequencing of faecal samples

High-throughput DNA sequencing techniques have revolutionised the investigative field of identifying the constituents of the microbiome compared to traditional culture-based techniques. Current methods generally involve amplifying and sequencing target microbial DNA regions, and then identifying organisms and assessing diversity based on the similarity of sequences compared to reference microbial genomic databases. The 16S ribosome gene (16S

rRNA) is often targeted in bacterial compositional studies as it contains nine variable regions between conserved regions, with the variable regions differing between genus and species. 16S rRNA sequencing differs from whole-genome shotgun sequencing, with the latter sequencing all the genes within the sample and consequently can provide functional information about the microbiome. Most intestinal microbial studies have used faecal samples, as obtaining samples is safer for the patient and more practical. One study has shown similarity in genera between the faecal and mucosal-associated bacterial communities, but other studies have shown substantial differences between these areas (288).

2.3.1 Illumina's 16S Metagenomic Sequencing

Illumina's 16S Metagenomic Sequencing Library Preparation Protocol (289) was used to prepare the sample libraries. The V1 and V2 variable regions were amplified using the primers detailed in Table 2.1. The forward primer mix was made up of four different forward primers, mixed at a ratio of 4:1:1:1 (28F-YM:28F-Borrellia:28FChloroflex:28F-Bifdo).

Primer name	Primer sequence
28F-YM (forward primer)	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAGTTTGATYMTGGCTCAG
28F-Borrellia (forward primer)	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAGTTTGATCCTGGCTTAG
28FChloroflex (forward primer)	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAATTTGATCTTGGTTCAG
28F-Bifdo (forward primer)	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGGTTCGATTCTGGCTCAG
388R (reverse primer)	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGCTGCCTCCCGTAGGAGT

Table 2.1 - Illumina MiSeq primers used for 16S rRNA gene sequencing – adapted from (90)

An Illumina MiSeq platform (Illumina Inc., Saffron Walden, UK) was used at St Mary's Hospital to perform 16S rRNA sequencing utilising the MiSeq Reagent Kit v3 (Illumina) and paired-end 300bp chemistry. The MiSeq platform performed clonal amplification, sequencing of the variable regions of the 16S rRNA gene, and then classification of faecal bacteria DNA within the sample.

The protocol involved initially performing PCR to amplify the V1 and V2 regions out of the faecal DNA samples using the above primers (Table 2.1). This was followed by the first PCR clean up step using AMPure XP beads to clean the 16S V1 and V2 amplicon from free primers and primer dimer species. Index PCR was then performed where dual indices and Illumina sequencing adapters were attached, and then another clean up and normalisation step utilising the SequalPrep Normalization Plate Kit (Life Technologies, Paisley, UK). Pooled libraries were then denatured before sequencing.

This protocol identified organisms from the resulting V1 and V2 amplicon utilising a 16S rRNA database - the Greengenes database (http://greengenes.lbl.gov/) - and results given at several taxonomic levels: kingdom, phylum, class, order, family, and genus. NEBNext Library Quant Kit for Illumina (New England Biolabs, Hitchin, UK) was utilised to quantify sample libraries.

2.3.2 Data processing

16S rRNA gene sequence data generated by the Illumina MiSeq platform was processed utilising the Mothur package (290) keeping to the MiSeq SOP Pipeline (291). During processing there was pairing of forward and reverse reads, and low-count and duplicate reads were removed, along with sequences containing ambiguous bases. The silva bacterial database was used to carry out sequence alignment (292), and the Wang method (293) was used to classify the sequences using the RDP database reference sequence files.

2.3.3 Diversity analysis

Different metrics can be used to describe the diversity of organisms within a sample. For microbiological studies, alpha diversity is used to measure the compositional diversity within a sample, and beta diversity measures compositional differences between groups of samples.

Alpha diversity includes richness, which reflects the number of different bacteria, and evenness, that measures how uniformly distributed the different taxa are within a sample. Richness can be represented by the Observed OTU count and Chao1 index, whereas the Shannon index and Fisher's index account for both richness and evenness. Calculations were performed within Mothur for alpha diversity (290), and then Wilcoxon matched-pairs signed rank test was used within GraphPad Prism statistical analysis software programme version 8.0.2 to assess for statistically significant changes between comparison groups. A p value of less than 0.05 was considered significant.

For beta analysis, a UniFrac weighted distance matrix was created from Mothur was used to generate a non-metric multidimensional scaling (NMDS) plot and PERMANOVA p-values were used to assess significance, and these data were analysed using the Vegan library within the R statistical package (294). Multidimensional scaling (MDS), or principal coordinate analysis (PCoA), is a visual representation of dissimilarities between sets of samples (Kruskal and Wish ref, 1978). More similar samples are closer on the graph compared to samples which are less alike. Non-metric multidimensional scaling uses rank orders as opposed to absolute abundances of OTUs, and so can reduce complex multidimensional data allowing it to be more easily plotted and visualised (295). For microbial diversity analysis, both unweighted and weighted UniFrac distances are used. Unweighted Unfrac distances are based on phylogenetic branches and OTU count, whereas weighted UniFrac distances include phylogenetic branches, OTU count and abundance (296).

2.3.4 Compositional analysis

Direct comparison of different bacterial abundance was made using bar charts in Excel at phylum, class, order, family and genus levels. The Statistical Analysis of Metagenomic Profiles software (STAMP) package was utilised to assess for statistically significant differences in bacterial composition between different groups using White's non-parametric t-test with Benjamini-Hochberg False Discovery Rate (FDR), and extended error bar plots were generated.

2.4 Urinary and faecal water NMR spectroscopy

2.4.1 NMR spectroscopy

Over the last few years nuclear magnetic resonance (NMR) spectroscopy has increasingly become the predominant technique for determining the structure of organic compounds. The NMR phenomenon can be simply described as applying a magnetic field to a sample, and then subjecting it to energy in the form of radiofrequency radiation. At the appropriate frequency, this causes the nuclei of the sample to absorb the energy, and then the duration and nature of energy dissipation from the nuclei provides information about that constituents of that sample (297).

Nuclei of elemental isotopes have a positive charge, and many act like they are spinning – called nuclear spin (*I*). In many atoms (for example carbon-12) these spins are paired against each other, and so there is no overall spin ($I = 0$). However, in atoms where there are an odd number of neutrons plus protons, or both the number of neutrons and protons are odd, then there is an overall net spin (*I* = half integer or integer spin respectively). As the nucleus contains spinning charges, a magnetic field is generated. In an external static magnetic field B_0 and at thermal equilibrium, two spin states can exist – a ground state (parallel to B_0), and an

excited state (anti-parallel to B_0), with only infrequent flipping of nuclei between states. Applying bursts of energy in the form of radiofrequency pulses (B_1) that equates to the energy difference of the two nuclear spin energy states causes much more flipping between the two energy states, and this flipping is referred to as the resonance process. The excited spin state energy is then released after the radiofrequency (RF) pulse is removed and the nuclei go back to an equilibrium state, and the released radiofrequency energy can be detected by a coil of wire tuned to the correct frequency. The signal is shown as a free induction decay (FID) following amplification. The FID is characterised by two major spin relaxation processes: the spin-lattice relaxation (T1) which is the duration for the nuclei to return to their original spin, and the spin-spin relaxation which is the amplitude of the relaxation in the transverse plane (T2) (297-299). Using a technique called Fourier Transformation, FIDs are mathematically simplified and transferred into the Frequency domain, and normally presented in the form of a one-dimensional (1D) spectrum – an example of a 1D NMR spectrum is shown in Figure 2.1.

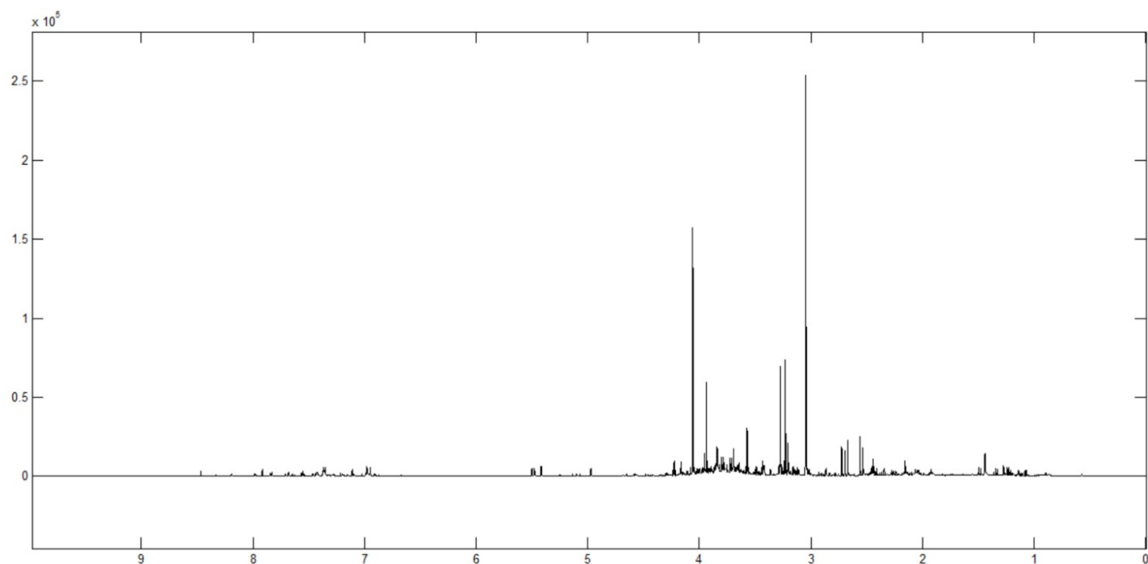

Figure 2.1: Example of a NMR spectrum

The NMR spectrum (Figure 2.1) shows the intensity on the y axis, and resonant frequency (the difference between the two spin states) as a chemical shift δ (expressed in parts per million) on the x axis, which is compared to a reference signal. The reference signal used in this project is 3-trimethylsilyl-1-1-(2,2,3,3,-2H4) propionate (TSP), which is chemically non-reactive.

The magnetic field experienced by particular protons in a molecule are influenced by the motions of nearby electrons, and so different positioned protons resonate at slightly different frequencies at different applied fields allowing specific proton environments within molecules to be identified. This effect is called 'chemical shift', and the nucleus of a specific chemical group shielded by a higher electron density will have a lower chemical shift, and vice versa. All nuclei experience a local magnetic field created by the surrounding electrons along with the static magnetic field B_0 and the applied field B_1. This local magnetic field depends on the exact electron environment, a function of the chemical structure of the molecule, and influences the degree of shielding or enhancement this has on the amount of RF energy absorbed. NMR peaks corresponding to specific molecules can be split by a phenomenon called 'spin-spin coupling', which is caused by the interaction of neighbouring protons in more than one kind of environment within a molecule interacting with each other and affecting the magnetic energy of the other protons. These split peaks can help identify molecules within a spectrum and add to the diagnostic ability of NMR. Identification of molecules within complex biological samples is possible using databases that contain information about chemical shifts and the number of peaks relating to each specific metabolite. As the intensity of the peak is proportional to the number of nuclei generating that signal, the area under a peak can be integrated to determine relative quantification of the corresponding metabolite (300).

2.4.2 Preparation of urine and faecal samples

A standardised method (301) was used to prepare urine and faecal samples using laboratory agents supplied by Sigma-Aldrich, USA, unless otherwise stated. 200µL of PBS (pH 7.4) was added to 400µL of urine or faecal water to stabile the pH. TSP and deuterium oxide (D_2O) were already mixed into PBS, with the former to act as a reference standard, and the latter as a field lock. For each sample, the above mixture was centrifuged for 5 minutes at 12,000g, and then 550µL was transferred to sterilised 5mm NMR tubes.

2.4.3 NMR Data acquisition and pre-processing

All spectra were phased, calibrated (using TSP), and baseline corrected automatically in Topspin. Spectral data was imported into MATLAB (version 2017a, The Mathworks, Inc.; Natwick, MA) using in-house scripts before spectral regions containing redundant information were removed. This included peaks corresponding to water and TSP, at 4.6 – 4.85 ppm and -0.2 to 0.2 ppm respectively. Prior to modelling, all data was aligned using an in-house automatic alignment function. Spectral data was then normalized using a probabilistic quotient approach.

Multivariate statistical analysis was employed to investigate differences between study groups. This was done using SIMCA (version 15, Umetrics, Sweden). Principal components analysis (PCA) was first carried out using univariate scaling to allow for the identification of any outliers and initial clustering based on principle components. Following this, orthogonal projection to latent structures discriminant analysis (OPLS-DA) was used to identify metabolites responsible for group membership; a maximum of two orthogonal components was used as to avoid over fitting the model. The discriminatory power of each OPLS-DA model was then validated by seven-fold cross-validation, providing a Q2 and R2 value representative

of predictive ability and degree of fit respectively. Finally, a CV-ANOVA with 1000 permutations was run to ascertain the significance of each model and obtain a p value.

Peak integral values for selected metabolites were obtained using an in-house Matlab script. Using the integral values, a univariate statistical approach was used to compare the relative concentration of metabolites of interest between the three time points. GraphPad Prism statistical analysis software programme version 8.0.2 was used to perform the appropriate univariate statistical test and corrected this for multiple comparisons. The panel of metabolites assessed were identified based on the specific hypothesis for each study.

2.4.4 Statistical Total Correlation Spectroscopy Editing (STOCSY-E)

Statistical Total Correlation Spectroscopy Editing (STOCSY-E) of ^{1}H NMR Spectra acquired from biofluids is a procedure that enables deconvolution of drug from endogenous metabolites. This procedure involves the identification of structurally correlated drug metabolic resonances, and then this is utilised to scale the 1H NMR spectra across regions containing the correlating drug peaks, and then a modified set of spectra containing reduced drug metabolite resonances is produced and allows reconstruction of potential endogenous peaks in drug metabolite peak regions. The STOCSY-E procedure contains fundamentally two steps; the identification of signals resulting from drug related compounds, and then selective scaling and background correction across the identified regions (302).

Chapter 3: Examining the differences in urinary metabonomic profiles of a real-life IBD population and relating it to changes in the faecal microbiome.

3.1 Summary

The intestinal microbiome has been extensively studied in IBD to further understand the complex pathogenesis of this condition, with microbiomic changes thought to be involved in propagating the abnormal immune response (33). Changes have been reported in intestinal microbial associated metabolites within a range of biological samples to further understand the role of microbiome in IBD, and to assess the potential of metabolic profiling in disease phenotyping and treatment response (156). Urine has been widely studied in IBD metabolomic research due to its ease in collection and handling, and extensive metabolic data including differences in microbial associated metabolites between IBD patients and healthy controls (161, 207, 209-211). These studies have been limited to subjects with no comorbidities, and so not reflecting a real-life clinical population.

This study assessed the faecal microbiome and urinary metabolome to examine whether the urinary metabolome was still distinct in a real-life population when compared to non-IBD controls, and whether changes in the metabolome could be linked to changes seen in the faecal microbiome.

Urine samples were acquired from 117 Crohn's disease, 98 UC, and 100 control subjects for ^{1}H NMR spectroscopy analysis. Contemporaneous faecal samples were obtained from the same subjects recruited for urine metabonomic analysis in 60 Crohn's disease, 43 UC, and 45 control subjects – faecal microbiomic analysis using 16S rRNA sequencing was restricted to subjects with no comorbidities and healthy controls as an insufficient number of faecal samples were obtained from IBD and non-IBD patients with comorbidities to allow for meaningful analysis in these cohorts.

Faecal 16S rRNA analysis in this study showed a reduction in alpha-diversity in the Crohn's disease cohorts, and compositional changes included an enrichment of *Bacteroidetes* and a lower abundance of *Clostridia* species. Initial multivariate ^{1}H NMR analysis of urine samples demonstrated that the presence of 5-aminosalicylates (5-ASAs) as the principal component driving separation between IBD and control cohorts, an issue that has been previously reported (215). Statistical total correlation spectroscopy editing (STOCSY-E) (302) was unsuccessful in deconvoluting the distortion of 5-ASAs on the NMR spectrum, and so subjects taking this medication were removed from further analysis. Multivariate separation between IBD and control subjects was then driven by microbial related metabolites hippurate, 4-cresol sulfate, and formate, the tricarboxylic acid (TCA) cycle metabolite citrate, the amino acid alanine, and these changes were consistent with previously reported studies (156). Targeted analysis showed consistent differences in hippurate and 4-cresol sulfate between IBD cohorts relative to controls, likely associated with reduced abundance of *Clostridia* species seen in faecal samples analysed with 16S rRNA sequencing. Overall, changes seen in previous studies appear to be consistent in a real-life population, and some of these changes can be attributed to corresponding differences in the faecal microbiome.

3.2 Introduction

Dysbiosis is thought to be an important part of the pathogenesis of inflammatory bowel disease. IBD related dysbiosis has been extensively investigated, with previous studies showing a loss of microbial diversity (303), and specific bacterial compositional changes. There has been variation in the previously reported microbial changes associated with IBD, which likely relates to different methodology and analytical techniques, however there has been some consistency in findings across several studies. At phyla level, an increase in *Bacteroidetes* and *Proteobacteria* (particularly *Escherichia coli*), and a loss of *Firmicute*

species have been associated with IBD. (75-77) Within the loss of *Firmicutes* phylum, a loss of the butyrate-producing *Faecalibacterium prausnitzii, Roseburia homonis* and *Clostridium groups IV* and *XIVa* have been reported. A loss of B*ifidobacterium* and an enrichment of *Fusobacteria* have also been observed in IBD (52, 70).

The intestinal microbiome has been shown to influence the metabolome in different biofluids and tissues (80, 89). Consequently, multiple studies have been conducted to examine the influence of IBD on these metabolomes to further understand the pathogenesis of this disease, and to investigate its use in stratifying patients into different disease phenotypes and treatment outcome groups (156). Urine has been widely studied in IBD metabonomic research due to its ease in collection, and handling and processing in the laboratory. Urine also provides extensive metabolic information including the endpoint profiles of microbial and host-microbial metabolism (161).

^{1}H NMR spectroscopy has been the analytical platform predominantly used in IBD urinary metabonomic studies (161, 207, 209-211). This platform involves the application of radiofrequency pulses to a sample within in a magnet field causing molecules to resonate between two states to determine the molecular constituents and their relative abundance within the sample (297). The advantage of ^{1}H NMR spectroscopy over mass spectrometry is high throughput analysis through comparatively simple sample preparation and speed of analysis. NMR data has high reproducibility but is less sensitive than mass spectroscopy (160).

Urinary ^{1}H NMR studies have shown that intestinal bacterial associated metabolites have been discriminatory when comparing IBD and healthy control cohorts (161, 207, 209-211). Williams et al. (161) published the first study using urinary ^{1}H NMR spectroscopy and observed differences between the urinary metabolomes of Crohn’s disease and UC relative to controls, and Crohn’s disease compared to UC. This study excluded IBD patients with other

comorbidities, and subjects taking 5-aminosalicylates and paracetamol had to be excluded from the final analysis as these xenometabolites were the principal components driving initial multivariate models. Following removal of these subjects, intestinal microbial associated metabolites were important in distinguishing IBD profiles from controls. A later study by Bjerrum et al. (211) did not observe differences in UC urinary NMR profiles, but this was following the removal of the aromatic region of the spectrum to account for the effect of 5-ASA resonances on multivariate analysis – this area also contains resonances from microbial associated metabolites which likely accounts for this study's findings (215).

There are several urinary metabolites that have shown to be discriminatory in IBD when compared to healthy controls (156, 161, 207, 209-211). These include a reduction in excretion of the microbial related metabolites hippurate, 4-cresol sulfate, formate and trigonelline (161, 207, 209, 210, 304, 305). Lower excretion of short chain fatty acids (SFCAs) acetate and butyrate (210, 306), and increased excretion of the amino acid alanine (217, 220).

Patients with IBD within the normal clinical setting will often have co-existing health conditions; 56 % of IBD patients had one additional significant comorbidity in a survey of 91 patients from three consecutive combined IBD outpatient clinics at St Mary's Hospital, Paddington, London (307). Despite this, IBD urinary metabonomic research to date either excludes subjects with comorbidities, or the presence of such co-existing conditions are not reported in the publications.

This is of importance as intestinal microbial associated urinary metabolites have been shown to be influenced by other common conditions including diabetes mellitus, asthma, and cardiovascular disease; for instance, altered renal excretion of the intestinal microbial related metabolites hippurate and dimethylamine have been seen in patients with type 2 diabetes mellitus (224, 225) and hypertension (234, 308), and different levels of hippurate and

trimethylamine N-oxide (TMAO) have been shown in asthma (229). TMAO has also been associated with cardiovascular disease (309, 310).

This study examined whether urinary metabolic profiling using ^{1}NMR spectroscopy can be applied to a real-life clinic population, and specifically included subjects with comorbidities, and assessed whether changes in the urinary metabolome specific to IBD could be associated with changes in the faecal microbiome.

3.3 Aims and hypothesis

The first part of this chapter examines the IBD associated faecal microbiome by comparing it to healthy control subjects to assess whether differences can be linked with changes seen in the urinary metabolome.

The second part of this chapter examines whether differences in the urinary metabolome associated with inflammatory bowel disease can be seen in a real-life population of subjects, including those comorbidities that have been excluded from previous studies.

Hypothesis: Urinary metabolic profiles associated with IBD are specific to this condition and will therefore present when applied to a cohort of subjects representing a real-life clinic population, and this population will include subjects with comorbidities.

3.4 Methods

3.4.1 Ethical approval

This study had ethical approval from Imperial College Healthcare NHS Trust Research and Ethics Committee (Ref: 13/LO/1867).

3.4.2 Study subjects

IBD and control subjects were recruited from gastroenterology, diabetes, cardiology, respiratory clinics from Imperial College Healthcare NHS Trust (ICHT), at the St Mary's Hospital and The Hammersmith Hospital sites, and London North West University Hospitals NHS Trust, at the Ealing Hospital site. Healthy controls were recruited from subjects working at ICHT and Imperial College London, and non-IBD controls were recruited from diabetes and chest clinics at St Mary's Hospital. 117 Crohn's disease patients, 98 UC and 100 control subjects were recruited, with detailed clinical data, dietary and medication information, obtained at the time of sample collection.

Urine samples were collected from all study subjects to assess the utility of metabonomic analysis in a wider population of patients than in previous studies to reflect a more real-life clinic population. Contemporaneous faecal samples were collected from the same cohort of subjects but restricted to IBD patients with no comorbidities and healthy controls – this was to make the groups more homogeneous so as to assess whether changes in the faecal microbiome of IBD patients could be linked to changes in discriminatory urinary metabolites associated with this disease. It was then planned to examine the urinary metabolome in comorbid IBD subjects and non-IBD subjects, but an insufficient number of faecal samples were collected to allow this analysis.

Ethnicity of subjects were separated into Caucasian, South Asian, and others. Caucasian subjects were defined as individuals who were white and of European decent. South Asians included subjects with ethnic-cultural heritage from the following countries: Afghanistan, Bangladesh, Bhutan, India, Maldives, Nepal, Pakistan, and Sri Lanka. Study subjects of 'other' ethnicity were defined as all subjects of another ethnic-cultural heritage outside of the above defined Caucasian and South Asian groups. The aim was to recruit equal proportions of

subjects from each of the above ethnic definitions, and of particular note, the sites for recruitment serve a large South Asian population, and so it was important to account for any confounding related to this ethnic group.

IBD diagnosis was based on clinical, radiological, endoscopic and histological data taken from the patient's paper and electronic notes, and from the pathology, radiology and endoscopy electronic reporting systems.

Exclusion criteria included subjects with IBD unclassified (IBDU), stomas, and currently pregnant. Subjects who had recently taken antibiotics (within 8 weeks), pre- or probiotics, or on liquid or treatment diets were also excluded. The diagnosis of IBDU was made based both on the diagnosis documented in patients' health records, but also on review of their clinical, histological, endoscopic and histological data.

3.4.3 Demographic and clinical data analysis

To assess for statistically significant differences in demographic and clinical data between cohorts, the Mann-Whitney U test and Fisher's exact tests were used. To correct for multiple comparisons, a Holm-Bonferroni adjustment was applied. For non-parametric data, a Chi-squared test (X2) was used.

3.4.4 Sample collection

Faecal samples were collected using spoon cap sample containers (Sigma-Aldrich, USA), and were then separated into microcentrifuge tubes (Ependorff, Germany) prior to being frozen at -80 ^{o}C on the same day (within 12 hours of being produced). Samples were then defrosted at the time of faecal DNA extraction.

Random urine samples were collected in 30ml universal containers (Sigma-Aldrich, USA), and within 6 hours were centrifuged for 10 minutes at 13,000 rpm at 4 ºC. The supernatant was aliquoted off and transferred into a microcentrifuge tubes (Ependorff, Germany) and stored at -80 ºC until used for NMR analysis.

3.4.5 Faecal microbiome analysis

The PowerLyzer PowerSoil DNA Isolation Kit (Mo Bio, Carlsbad, CA, USA) was used to perform DNA extraction on faecal samples using the manufacturer's protocol. For each sample, 250 mg of faeces and an in-house additional bead beating step was used (90). The extracted DNA was then stored at -80ºC. Illumina's 16S Metagenomic Sequencing Library Preparation Protocol (289) was used to prepare the sample libraries. An Illumina MiSeq platform (Illumina Inc., Saffron Walden, UK) was used to perform the sequencing using the MiSeq Reagent Kit v3 (Illumina) and paired-end 300bp chemistry.

16S rRNA sequencing data was then analysed using the Mothur package following the MiSeq SOP Pipeline (290). Sequence alignment was performed using the Silva bacterial database, and the Wang method using the RDP database was used for classification of sequences (292). Calculations were performed within Mothur for alpha diversity (Shannon diversity index, H'), and Wilcoxon matched-pairs signed rank test using GraphPad Prism statistical analysis software programme version 8.0.2 was used to performed to assess for statistical significance between time points. The Shannon index creates a number (between 0 and 1) that summarises the species diversity within a sample, and is based on the richness and evenness of species within the sample; richness is the total number of species within a sample, and evenness reflects how uniformly distributed the different species are within samples (311). Beta diversity, a measure of compositional similarity between samples, was assessed using the non-metric multidimensional scaling (NMDS) plot, and PERMANOVA p-values were generated using the UniFrac weighted

distance matrix. (294). The Statistical Analysis of Metagenomic Profiles software (STAMP) package (312) was used to assess for statistically significant differences in bacterial composition between subjects at different time groups using White's non-parametric t-test with Benjamini-Hochberg False Discovery Rate (FDR).

3.4.6 Metabonomic analysis

Urine samples were analysed using 1D ^{1}H NMR spectroscopy. All spectra were phased, calibrated, (using TSP) and baseline corrected automatically in Topspin. Spectral data was imported into MATLAB (version 2017a, The Mathworks, Inc.; Natwick, MA) using in-house scripts before spectral regions containing redundant information were removed. This included peaks corresponding to water and TSP, at 4.6 – 4.85 ppm and -0.2 to 0.2 ppm respectively. Prior to modelling, all data were aligned using an in-house automatic alignment function. Spectral data was then normalized using a probabilistic quotient approach.

Multivariate statistical analysis was used to investigate differences between study groups. This performed using SIMCA (version 15, Umetrics, Sweden) and Matlab (version 2017a, The Mathworks, Inc.; Natwick, MA). Principal components analysis (PCA) was carried to identify of outliers and initial clustering based on principle components. Univariate scaling (scaling weight = inverse standard deviation of variable) was applied to allow for smaller variations in data during multivariate analysis. Outliers with a distance from the model (DModX) that exceeded the critical value (Dcrit) were excluded. The chemical shift of these samples was identified using a loadings plot, and the subsequent driving metabolite could be identified. A 7-fold cross validation was undertaken in each model.

Supervised multivariate analysis was performed using orthogonal partial least squares – discriminative analysis (OPLS-DA). This generates a R2 value and a Q2 values. R2 represents the proportion of variance for the dependent variables that is explained by the independent

variables within the model. Q2 is used to validate the model and estimates its predictability using cross-validation. This is done taking out 1/7th of the data and building a new model using the remaining 6/7th of the data, and then assesses how well the new model predicts the left-out data. This is repeated until every 1/7th of the data in the model is predicted in this manner. All the predicted data is then compared with the original data to generate this value. The higher the Q2, the better the predictability of the model. Positive Q2 values undergo permutation testing or CV-ANOVA to calculate a p value (values less than 0.05 were considered significant). The Human Metabolome Database and Chenomx Profiler (Chenomx NMR Suite 8.1) were used to identify metabolites contributing to the model from the loadings plot, and correlating peaks from the same metabolite were confirmed using Statistical evidence from STOCSY Matlab (version 2017a, The Mathworks, Inc.; Natwick, MA).

For targeted analysis, peak integral values for selected metabolites were obtained using an in-house Matlab script (90). These metabolites were identified from a mean spectrum of all the samples, and a mean integral of a peak from each targeted metabolite was used to calculate a relative abundance. Using the integral values, a univariate statistical approach was employed to compare the relative quantity of metabolites of interest between cohorts. GraphPad Prism statistical analysis software programme version 8.0.2 was used to perform a Mann-Whitney U test for univariate analysis. A Benjamini-Hochburg calculation was applied to adjust for multiple comparisons.

Targeted analysis was performed on urine metabolites that were either demonstrated in previous studies to be consistently discriminative in IBD (161, 207, 209, 210). These are as follows: 4-cresol sulfate, formate, hippurate, methylamine, trigellonine, citrate, succinate and alanine – see Table 3.1.

Metabolite	Formula	Multiplicity	Chemical shift (ppm)	ppm range used for integrals (ppm)
p-cresol	C_7H_8O	d d s	7.13 6.82 2.25	2.256 to 2.266
formate	CH_2O_2	s	8.46	8.460 to 8.470
hippurate	$C_9H_9NO_3$	d t t	3.97 7.55 7.64	3.965 to 3.982
methylamine	CH_5N	s	2.59	2.582 to 2.595
trigonelline	$C_7H_7NO_2$	s m m s	9.11 8.82 8.07 4.43	4.420 to 4.442
acetate	$C_2H_4O_2$	d q	1.49 3.79	3.744 to 3.812
butyrate	$C_4H_8O_2$	t m t	2.14 1.54 0.88	2.130 to 2.152
citrate	$C_6H_8O_7$	d d	2.55 2.67	2.525 to 2.570
succinate	$C_4H_6O_4$	s	2.39	2.280 to 2.412
alanine	$C_2H_3O_2$	s	1.92	1.475 to 1.500

Table 3.1: Urine metabolites selected for targeted univariate analysis. Table shows chemical formula, and multiplicity and chemical shift for their $_1$H NMR peaks. Chemical shifts marked in bold indicate peak integration used in this study. s=singlet, d=doublet, t=triplet, m=multiplet

3.5 Results

3.5.1 Subject characteristics of all study subjects

117 Crohn's subjects, 98 UC subjects, and 100 non-IBD control subjects were analysed.

Subject characteristics are summarised in Table 3.2 below.

	CD, *n = 117*	UC, *n = 98*	Controls, *n = 100*
Age (mean, years)	48 (IQR 24)	49 (IQR 25)	53 (IQR 15)
Sex (M : F) *(%)*	50 : 67 *(43 : 57)*	48 : 50 *(49 : 51)*	56 : 44 *(56 : 44)*
Ethnicity (Cau : SA : Oth)* *(%)*	73 : 29 : 15 *(62 : 25 : 13)*	56 : 32 : 10 *(57 : 33 : 10)*	42 : 47 : 11 *(42 : 47 : 11)*
BMI (median, kg/m^2)	23.9 (IQR 6.3)	24.7 (IQR 7.6)	26.3 (IQR 6.7)
Disease location** *(%)*	L1: 20 *(17)* L2: 62 *(53)* L3: 35 *(30)*	E1: 24 *(24)* E2: 41 *(42)* E3: 33 *(34)*	- - -
Disease activity†: *(%)*	Remission: 98 *(84)* Mild: 9 *(8)* Moderate: 9 *(8)* Severe: 1 *(8)* Remission : active 98 : 19 *(84 : 16)*	Remission: 78 *(80)* Active: 10 *(10)* Severe: 10 *(10)* Remission : active 78 : 20 *(80 : 20)*	- - - - - -
Operation *(%)*	31 *(26)*	0 *(0)*	-
5 ASA *(%)*	43 *(36)*	70 *(71)*	-
Oral immuno-modulator *(%)*	49 *(42)*	34 *(35)*	-
Biologic *(%)*	23 *(20)*	5 *(5)*	-
Steroid *(%)*	4 *(3)*	9 *(9)*	1 *(1)*

Non-IBD medication *(%)*	66 *(56)*	51 *(52)*	47 *(47)*
Comorbidity: Total T2DM Asthma Hypertension / CVD Other *(%)*	 33 *(28)* 7 *(6)* 6 *(5)* 12 *(10)* 8 *(7)*	 41 *(42)* 11 *(11)* 8 *(8)* 11 *(11)* 13 *(13)*	 48 *(48)* 19 *(19)* 12 *(12)* 2 *(2)* 15 *(15)*

Table 3.2: Characteristics of study subjects. *Ethnicity; Cau = Caucasian, SA = South Asian, Oth = Other. **Montreal classification, CD L1 = ileal, L2 = colonic, L3 = ileo-colonic, UC E1 = proctitis, E2 = left sided, E3 = extensive. †CD HBI score, remission =<5 and active ≥5, UC SCCAI score, remission <5, active ≥5. ††Statistical comparison between groups, p value of <0.05 considered significant and are in bold.

3.5.2 Faecal 16S sequencing results

16S rRNA gene sequencing was performed on faecal samples from IBD subjects with no comorbidities and healthy controls. Subjects with comorbidities were excluded as there were not enough samples collected from this cohort to lead to meaningful analysis. Subjects taking 5-aminosalicylates or paracetamol were also excluded.

3.5.2.1 Faecal 16S sequencing of Crohn's disease subjects without comorbidities, and not taking 5-aminosalcylates

Subject characteristics are shown in table 3.3.

	n	Age (mean)	Sex (M : F)	Ethnicity* (Ca : SA : Oth)
CD *(%)*	*49*	49	24 : 25 *(49 : 51)*	29 : 18 : 2 *(59 : 37 : 4)*
HC *(%)*	*45*	53	26 : 19 *(58 : 42)*	16 : 27 : 2 *(36 : 60 : 4)*

Table 3.3: Subject characteristic of Crohn's disease (CD) and healthy control (HC) cohorts. Cohorts include subjects from all ethnic backgrounds, but those taking 5-aminosalicylates or have a comorbidity have been excluded. *Ethnicity: Cau = Caucasian, SA = South Asian, Oth = Other.

Analysis of alpha diversity, measured by the Shannon index, was reduced in the Crohn's disease cohort compared to the healthy controls – see Figure 3.1.

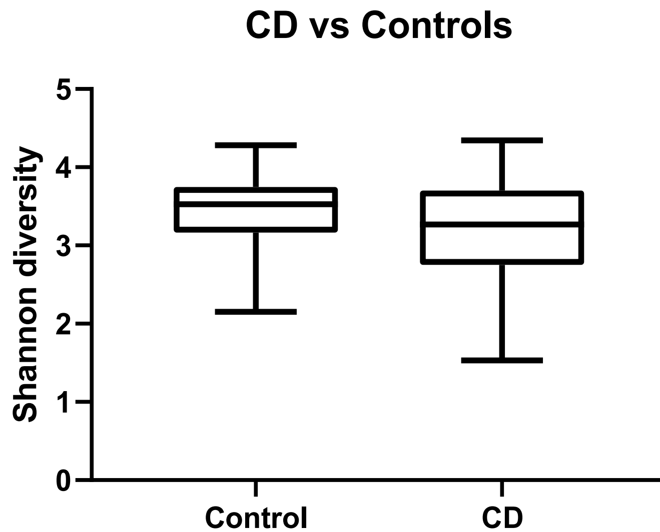

Figure 3.1: Alpha diversity as measured by the Shannon index between Crohn's disease (CD) and control cohorts, no 5ASAs. A two tailed Mann Whitney U test showed statistically significant difference between the two groups, $p = 0.024$.

Alpha diversity using the Shannon index was assessed in the UC cohort, assessing for any difference compared to healthy controls – no significant change in alpha diversity was shown in this comparison.

Compositional analysis comparing Crohn's disease subjects with healthy controls showed an increase in *Bacteroidetes* in the Crohn's disease cohort at class, order and family taxa levels. At family level there was also an increase in *Ruminococcaceae* in the Crohn's subjects – see Figure 3.2. At genus levels there was a reduction in *Clostridium XIV* and *Clostridium XVIII*. In the figure below, Crohn's disease cohorts are represented by the blue bars, and controls by the orange bars. This figure only demonstrates organisms at each taxanomic level that were found to have statistically significant differences between disease and control groups using White's

non-parametric t-test with a q-value < 0.05 (q value = p value corrected for multiple comparisons with Benjanmini-Hochber test).

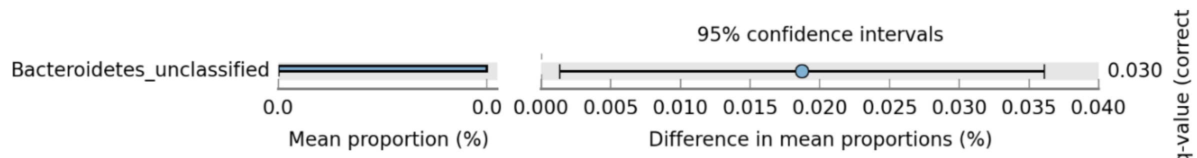

Figure 3.2(a) – Class

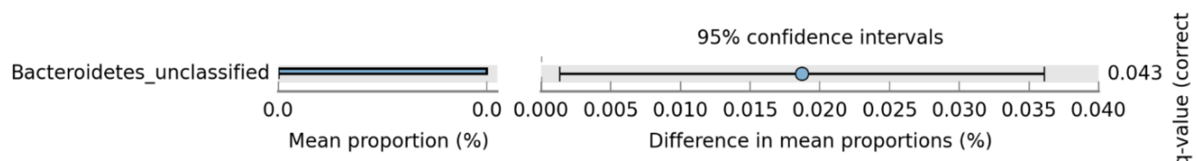

Figure 3.2(b) – Order

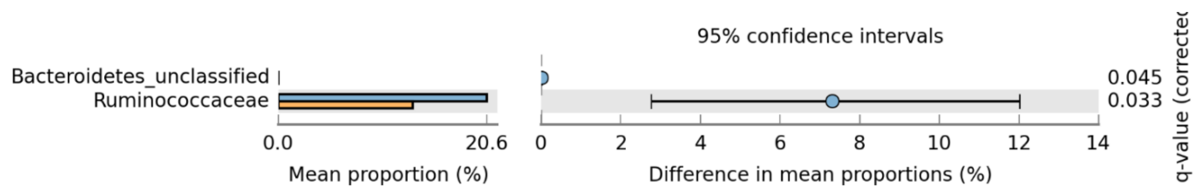

Figure 3.2(c) – Family

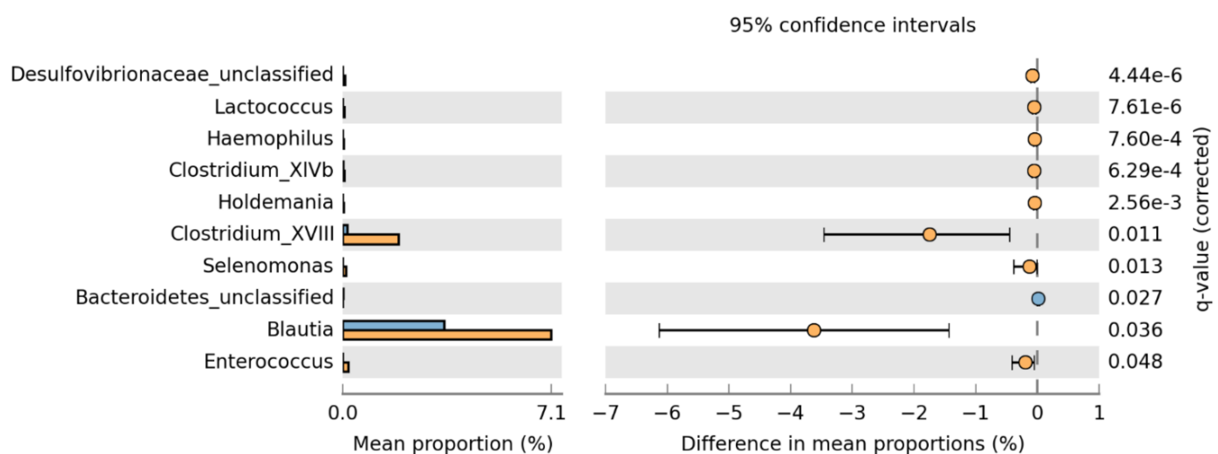

Figure 3.2(d) – Genus

Figure 3.2: Compositional analysis comparing Crohn's disease subjects with healthy controls. STAMP analysis using White's non-parametric t-test showed statistically different abundance of bacteria between Crohn's disease cohorts and healthy controls at (a) class, (b) order, (c) family and (d) genus taxa levels. Blue bars = CD cohort, orange = controls. Statistically significant differences between cohorts were not present at phyla level. q-value = p value corrected for multiple comparisons with Benjanmini-Hochber test.

3.5.2.2 Faecal 16S sequencing of UC subjects without comorbidities, and not taking 5-aminosalcylates

Subject characteristics are shown in table 3.4.

	n	Age (mean)	Sex (male : female)	Ethnicity* (Ca : SA : Oth)
UC *(%)*	*29*	49	16 : 13 *(55 : 45)*	18 : 10 : 1 *(62 : 35 : 3)*
HC *(%)*	*45*	53	26 : 19 *(58 : 42)*	16 : 27 : 2 *(36 : 60 : 4)*

Table 3.4: Subject characteristic of ulcerative colitis (UC) and healthy control (HC) cohorts. Cohorts include subjects from all ethnic backgrounds, but those taking 5-aminosalicylates or have a comorbidity have been excluded. *Ethnicity: Cau = Caucasian, SA = South Asian, Oth = Other.

There was no statically significant difference in alpha diversity between the UC cohort and healthy controls (p = 0.233). Compositional analysis comparing UC subjects with healthy controls (see Figure 3.3) showed an increase in *Bacteroidetes* at order level in UC patients, and a reduction of *Eubacteriaceae* and *Oxalobacteraceae* at order and family levels. UC patients have an increase in *Clostridiaceae* at family level, and an increase in *Clostridium_sensu_stricto* at genus level. Further differences at genus level included an increase of *Firmicutes_unclassified* in the UC cohort. In the figure below, UC cohorts are represented by the blue bars, and controls by the orange bars. This figure only demonstrates organisms at each taxanomic level that were found to have statistically significant differences between disease and control groups using White's non-parametric t-test with a q-value < 0.05 (q value = p value corrected for multiple comparisons with Benjanmini-Hochber test).

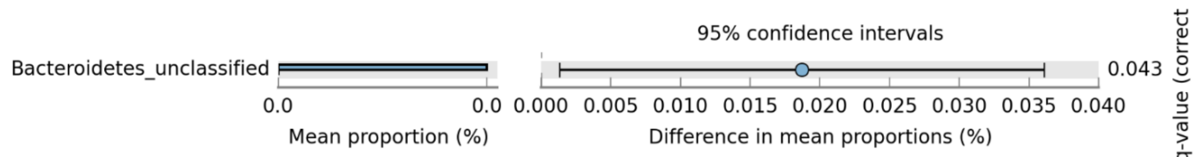

Figure 3.3 (a) – Order

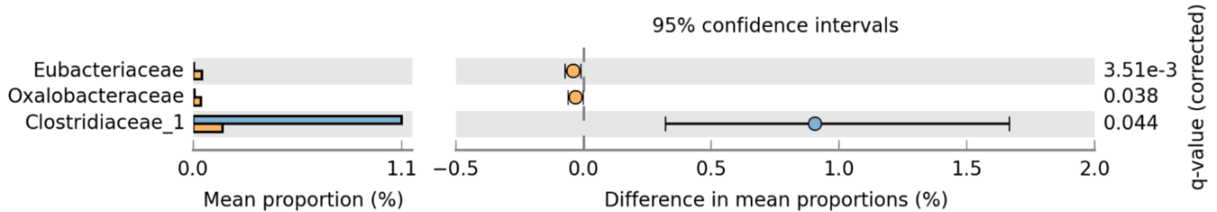

Figure 3.3(b) – Family

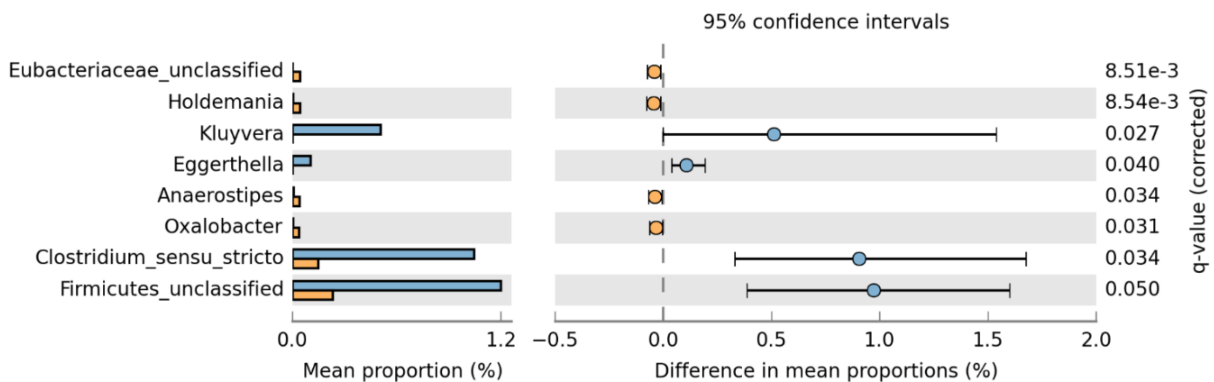

Figure 3.3(c) – Genus

Figure 3.3: Compositional analysis comparing UC subjects with healthy controls. STAMP analysis using White's non-parametric t-test showed statistically different abundance of bacteria between UC cohorts with no comorbidities and healthy controls at (a) order and (b) family and (c) genus taxa levels. Blue bars = UC cohort, orange = controls. Statistically significant differences between cohorts were not present at phyla, class and order levels. q-value = p value corrected for multiple comparisons with Benjanmini-Hochber test.

3.5.3 Metabonomic analysis of urine samples

This study used two approaches to analyse urine samples between different cohorts – supervised multivariate analysis using OPLS-DA, and targeted univariate analysis to compare relative abundance of microbial associated metabolites that have previously been shown to be affected in IBD.

3.5.3.1 Multivariate analysis of urine samples - all subjects (subjects with comorbidities included in IBD and non-IBD cohorts)

The first supervised multivariate model included all recruited subjects in the study, and compared IBD subjects with non-IBD subjects. This model included subjects with non-IBD comorbidities in the IBD cohort and non-IBD health conditions the control cohort. Figure 3.4 shows the OPLS-DA model comparing these groups. Table 3.2 in Section 3.5.1 gives details of the study characteristics.

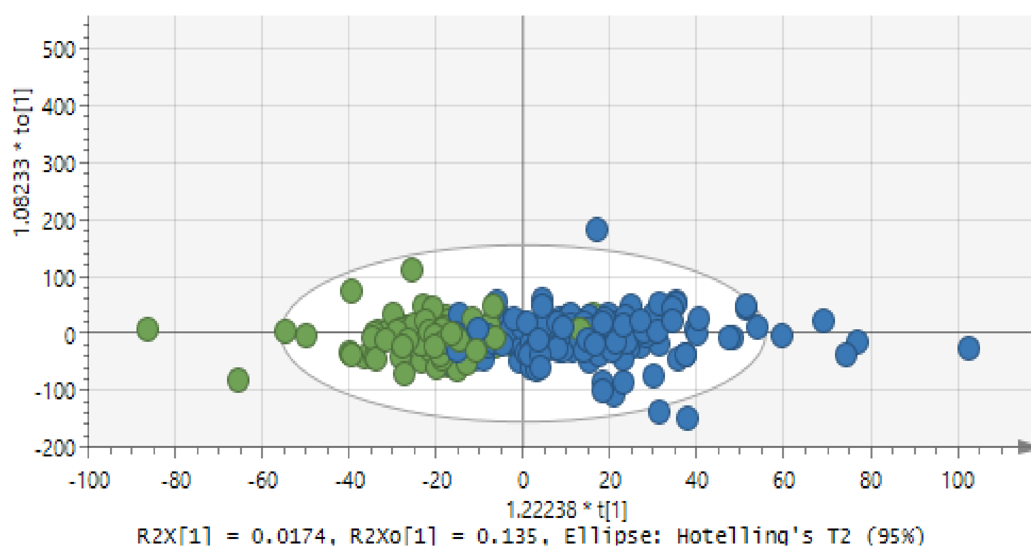

Figure 3.4: OPLSDA plot comparing IBD cohort with non-IBD cohort. Cohorts included subjects of different ethnic backgrounds, and subjects taking 5-aminosalicylates and those with comorbidities. IBD subjects are marked in blue, healthy controls are marked in green.

A covariance plot was used to identify metabolites driving the subsequent models. OPLS-DA cross-validated coefficients were plotted back scaled onto the original spectral data, and interpreted as a conventional NMR spectrum. Positive (upward pointing) and negative (downward pointing) signals denote metabolites with a relative increase and decrease in intensity respectively, with the strength indicated by the correlation coefficient (R2). A covariance plot of the aromatic region from the above OPLSDA model showed that the two

most significant metabolites driving separation are resonances from 5-aminosalicylates and hippurate– see Figure 3.5.

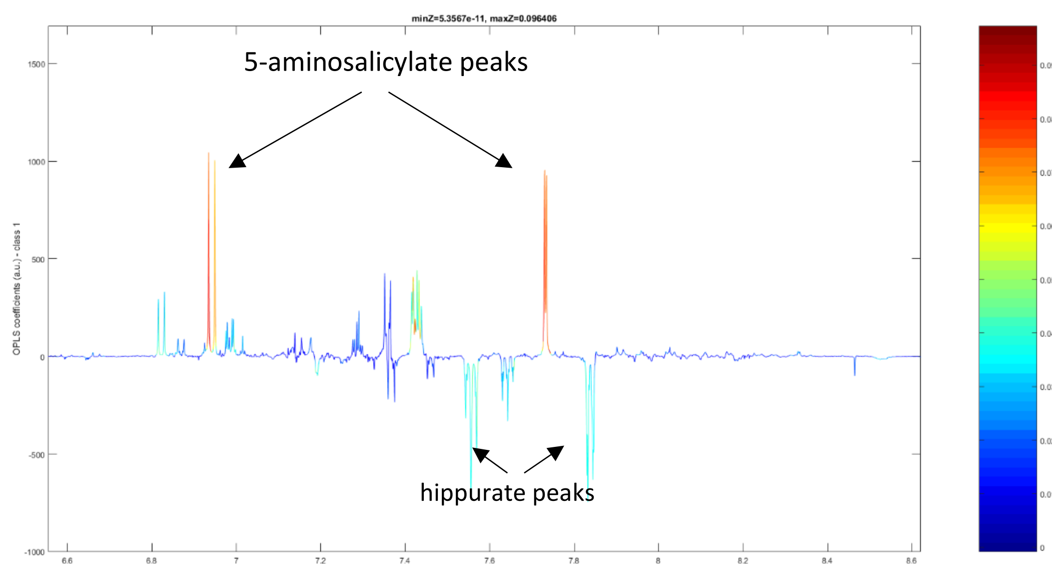

Figure 3.5: Covariance plot of aromatic region of OPLS-DA model comparing the IBD cohort with non-IBD cohort. Cohorts included subjects of different ethnic backgrounds, and subjects taking 5-aminosalicylates and those with comorbidities. This plot demonstrates that the main driving peaks in this OPLS-DA model correspond to 5-aminosalicylate and hippurate.

5-aminosalicylatess (5-ASAs) are known to affect the aromatic region of the urine spectra, a region of interest as it contains peaks corresponding to bacterial associated metabolites. 5-aminosacylate peaks are found at δ 2.17, 6.95, 7.44, 7.73 respectively (313). Statistical Total Correlation Spectroscopy editing (STOCSY-E), a technique that allows for the removal of metabolite peaks using statistical correlation between a driver peak and correlating peaks, was applied to remove resonances from xenometabolites. A driver peak at 2.17, corresponding to 5-ASA, was initially used to remove correlating 5-ASA peaks. However, this was unsuccessful owing to the significant overlap of 5-ASA peaks with endogenous metabolite peaks across this region of the spectra. Overlapping of peaks reduces the correlation between xenometabolite

peaks and their corresponding driver peak which means STOCSY-E cannot accurately reduce these peaks. Multiple driver peaks were then used across the spectra for both 5-ASAs, reducing the threshold of correlation that STOCSY-E uses to remove correlating peaks (from a default of 0.9 to 0.7), and manually aligning the spectra in these regions was also performed to improve separation between xenometabolite peaks with the aim of improving their correlation with the driver peak. Removal of xenometabolite peaks using STOCSY-E was also attempted using raw, unaligned data, and data that had only been aligned in the xenometabolite regions, but this did not allow for the successful removal of all xenometabolite peaks, and shifting of peaks across the spectra meant this data was also not useful for multivariate statistical interrogation.

3.5.3.2 Multivariate analysis of urine samples – subjects taking 5-aminosalicylates excluded

Subject characteristic comparisons following the removal of patients taking 5-aiminosalicylates and paracetamol is shown in Table 3.5. IBD and control cohorts include subjects with another comorbidity.

	CD, *n = 74*	UC, *n = 28*	Controls, *n = 100*
Age (mean, years)	43 (IQR 21)	53 (IQR 22)	53 (IQR 15)
Sex (M : F) *(%)*	31 : 43 *(41 : 56)*	13 : 15 *(46 : 54)*	56 : 44 *(56 : 44)*
Ethnicity (Cau : SA : Oth)* *(%)*	45 : 18 : 11 *(61 : 24 : 15)*	15 : 13 : 0 *(54 : 46 : 0)*	42 : 47 : 11 *(42 : 47 : 11)*
BMI (median, kg/m^2)	23.6 (IQR 7.3)	25.6 (IQR 9.1)	26.3 (IQR 6.7)
Disease location** *(%)*	L1: 14 *(19)* L2: 35 *(47)* L3: 25 *(34)*	E1: 7 *(25)* E2: 11 *(39)* E3: 10 *(36)*	- - -
Disease activity† *(%)*	Remission: 62 *(84)* Mild: 6 *(8)* Moderate: 6 *(8)* Severe: 0 *(0)* Remission : active 62 : 11 *(84 : 16)*	Remission: 22 *(79)* Active: 6 *(21)* Remission : active 22 : 6 *(79 : 21)*	- - - - - - -
Oral immuno-modulator *(%)*	36 *(49)*	6 *(21)*	-
Biologic *(%)*	26 *(35)*	3 *(11)*	-
Non-IBD medication *(%)*	39 *(53)*	14 *(50)*	47 *(47)*
Comorbidity: Total DM Asthma CVD / hypertension Other *(%)*	 19 *(26)* 4 *(5)* 4 *(5)* 4 *(5)* 7 *(9)*	 8 *(29)* 4 *(14)* 2 *(7)* 1 *(4)* 2 *(7)*	 48 *(48)* 19 *(19)* 12 *(12)* 2 *(2)* 15 *(15)*

Table 3.5: Characteristics of study subjects excluding patients on 5-aminosalicylates. *Ethnicity; Cau = Caucasian, SA = South Asian, Oth = Other. **Montreal classification, CD L1 = ileal, L2 = colonic, L3 = ileo-colonic, UC E1 = proctitis, E2 = left sided, E3 = extensive. †CD HBI score, remission =<5, mild = 5-7, moderate 6-18, severe >16, UC SCCAI score, remission <3, active 3-4, severe 5+.

Subject characteristic data after removal of subjects taking 5-aminosalicylates and paracetamol have been assessed for statistically significant differences and the results shown in Table 3.6. Comparisons between disease and control groups showed differences in age, ethnicity, BMI, and comorbidities. There were differences in the number of operations, IBD medications, and comorbidities between Crohn's disease and UC groups.

Subject characteristic comparisons - univariate analysis – p values				
	IBD : controls	CD : controls	UC : controls	CD : UC
n	*102 : 100*	*74 : 100*	*28 : 100*	*74: 28*
Age (median)[1]	**0.012**	**0.003**	0.704	0.112
Sex[2]	0.069	0.066	0.573	0.462
Ethnicity (Cau : SA : Oth)[*2]	**0.029**	**0.009**	0.107	**0.028**
BMI (median)[1]	**0.013**	**0.002**	0.960	0.058
Disease activity†[2]:	-	-	-	0.843
Operation[2]	-	-	-	**0.001**
Oral immuno-modulator[2]	-	-	-	**0.017**
Biologic[2]	-	-	-	0.061
Steroid[2]	-	-	-	0.519
Non-IBD medication[2]	0.481	0.457	0.779	0.807
Comorbidity[2]: Total DM Asthma CVD Other	 **0.002** **0.020** 0.127 0.157 0.262	 **0.003** **0.009** 0.136 0.114 0.420	 0.136 0.566 0.466 0.627 0.279	 0.514 0.137 0.739 0.541 0.578

Table 3.6 – Characteristic comparisons between subject cohorts after removal of subjects taking 5-aminosalicylates. [1]Mann-Whitney U test, [2]Chi-squared test, p value of <0.05 considered significant and are in bold. †HBI score or SCCAI score of ≥5.

Disease activity was assessed using HBI and SCCAI scores for Crohn's disease and UC respectively. At the time when recruitment commenced for this study, in early 2014, faecal calprotectin tests were just coming into routine monitoring use. Faecal calprotectin, a non-invasive marker of disease activity, was not used in this study but would have given an additional measure of disease activity to correlate with metabolomic and microbiome results.

Dietary and lifestyle factors known to potentially affect the urinary metabolome were also compared between cohorts and are shown in Table 3.7a and 3.7b. In the IBD compared to control cohorts, difference were seen in smoking (UC :C), meat consumed within the last 24 hours (CD : C), grapefruit (CD : C), mild (CD : C), and alcohol (IBD : C, and CD : C). Meat, berries and coffee consumption was different in the Crohn's disease compared to UC cohorts.

Lifestyle comparisons				
	IBD	CD	UC	controls
n	102	74	28	100
Vegetarian				
Smoker	17	5	12	2
Exercise	83	42	41	48
Consumed within the last 24 hours prior to producing samples:				
Meat	182	108	74	82
Fish	65	28	37	29
Cheese	127	70	57	48
Grapefruit	5	5	0	0
Cherries	4	0	4	1
Liquorice	13	5	8	3
Walnuts	18	14	4	15
Vanilla	26	14	12	7
Milk	158	80	78	81
Yoghurt	71	42	29	43
Berries	58	42	16	28
Carbonated drinks	110	61	49	44
Coffee	112	51	61	52
Tea	123	66	57	51
Herbal tea	48	23	25	17
ETOH	48	28	20	12

Table 3.7a – Lifestyle comparisons between subject cohorts.

Lifestyle comparisons - univariate analysis – p values				
	IBD : controls	CD : controls	UC : controls	CD : UC
n	*102 : 100*	*74 : 100*	*28 : 100*	*74: 28*
Vegetarian	0.184	0.291	0.227	0.830
Smoker	0.037	0.332	**0.004**	**0.031**
Exercise	0.115	0.071	0.383	0.373
Consumed within the last 24 hours prior to producing samples:				
Meat	0.552	**0.022**	0.264	**0.001**
Fish	0.823	0.398	0.191	0.398
Cheese	0.065	0.812	0.152	0.805
Grapefruit	0.124	**0.036**	0.999	0.038
Cherries	0.569	0.278	0.167	0.274
Liquorice	0.251	0.620	0.113	0.233
Walnuts	0.074	0.513	0.009	0.038
Vanilla	0.169	0.217	0.210	0.950
Milk	0.150	**0.034**	0.803	0.064
Yoghurt	0.086	0.285	0.050	0.328
Berries	0.850	0.215	0.048	**0.001**
Carbonated drinks	0.236	0.232	0.398	0.755
Coffee	0.988	0.216	0.145	**0.006**
Tea	0.302	0.425	0.311	0.796
Herbal tea	0.277	0.615	0.143	0.305
ETOH	**0.030**	**0.024**	0.108	0.537

Table 3.7b – Statistical analysis of lifestyle comparisons between subject cohorts. Chi square test, p value of <0.05 considered significant and are in bold.

Patient recall of dietary and lifestyle factors within and beyond 24 hours can be variable, and it can also be difficult to quantify some of these factors, and so accurately statistically assessing the influence of these important potential confounders can be challenging.

Multivariate models excluding subjects taking 5-aminosalicylates were able to discriminate IBD from non-IBD controls, and Crohn's disease from non-IBD controls - see Table 3.8. Models comparing UC and controls, and Crohn's disease and UC, were not significant.

IBD v non-IBD controls (C) [subjects on 5-ASAs excluded].						
Comparison cohorts	*n*	R2X	Q2	p value CV-ANOVA	Most significant metabolites driving model* (fold change)	Fold change
IBD : C	*202 (102 : 100)*	0.194	0.142	0.020	hippurate ↓ alanine ↓ citrate ↓ p-cresol ↓ PAG ↑ DMG ↓ formate ↓	0.72 0.87 0.89 0.86 1.21 0.91 0.91
CD : C	*174 (74 : 100)*	0.037	0.093	<0.001	hippurate ↓ alanine ↓ citrate ↓ p-cresol ↓ PAG ↑ DMG ↓ betaine ↓ formate ↓ sucrose ↑	0.69 0.88 0.86 0.90 1.19 0.92 0.88 0.89 1.23
UC : C	*128 (28 : 100)*	0.208	-0.157	NS†		
CD : UC	*102 (74 : 28)*	0.168	0.007	NS		

Table 3.8: OPLS-DA models comparing IBD and control cohorts, and includes subjects with comorbidities in IBD and non-IBD cohorts. *most significant metabolites driving separation as identified using a covariance plot, †NS, non-significant. Arrows denote whether the relative abundance of metabolite is higher or lower in the IBD cohort.

Covariance plots demonstrated the driving metabolites separating IBD and Crohn's cohorts from controls were hippurate, alanine, citrate, 4-cresol sulfate, phenyacetylglutamine (PAG), dimethylglcyine (DMG), betaine, and formate – see Table 3.8. Models comparing UC and healthy controls, and Crohn's disease and UC, were not significant.

When IBD and control subjects with a non-IBD health condition were excluded, similar discriminatory metabolites were identified driving the models – see Table 3.9. Supervised multivariate analysis could not construct models to separate UC from healthy controls and Crohn's patients from UC patients following the removal of subjects with non-IBD health conditions.

Further multivariate comparisons were made between IBD cohorts and controls, and similar to the above, discriminatory models could be generated for non-comorbid IBD and Crohn's disease cohorts compared to healthy controls, but not for UC and healthy controls, and between Crohn's disease and UC – see Supplementary Table 1. Discriminatory models could not be generated for comorbid only IBD cohorts and healthy controls, but these comparisons were likely underpowered – see Supplementary Table 2.

3.5.2.3 Targeted univariate analysis of urine samples

Targeted univariate analysis was performed to assess for statistically significant differences in metabolites of interest between cohorts. The first five metabolites selected for targeted analysis were related to intestinal bacterial metabolism or host-bacterial co-metabolism, and had shown to be significant in IBD in previous studies; these included 4-cresol sulfate, formate, hippurate, methalamine, and trigonelline. The TCA related metabolite citrate and succinate, and the amino acid alanine were also selected as these too have been consistently shown to be significant in IBD.

All IBD subjects including those with no comorbidities were compared with all controls (including those with comorbidities) – see Tables 3.9a and 3.9b. Patients taking 5-aminosalicylates were excluded, as 5-ASA signals interfered identifying targeted metabolite spectral peaks in the aromatic region preventing peak integral values being accurately selected for these metabolites.

Targeted analysis showed differences between the combined IBD cohort (Crohn’s disease and UC) and controls. There was a decrease in 4-cresol sulfate between all IBD cohorts and controls, and between Crohn’s disease and UC with a p value of less than 0.05. A statistically reduced reduction in hippurate excretion was present in IBD and Crohn’s disease cohorts compared to controls. Alanine excretion was reduced in all IBD cohorts compared to controls, citrate excretion was reduced in Crohn’s disease cohort compared to controls, and acetate excretion was increased in UC compared to controls, and reduced in Crohn’s disease compared to UC.

	All IBD and CD vs all controls (C)		
n		IBD : C *102 : 100*	CD : C *74 : 100*
	Microbial metabolites		
4-cresol sulfate	Median integral: Direction: p value =	12242 : 14724 ↓ : ↑ **0.001**	12833 : 14724 ↓ : ↑ **0.002**
formate	Median integral: Direction: p value =	11995 : 12383 ↓ : ↑ 0.262	11295 : 12383 ↓ : ↑ 0.673
hippurate	Median integral: Direction: p value =	196880 : 283119 ↓ : ↑ **0.004**	190145 : 283119 ↓ : ↑ **0. 002**
methylamine	Median integral: Direction: p value =	9318 : 9710 ↓ : ↑ 0. 945	9476 : 9710 ↓ : ↑ 0. 604
trigonelline	Median integral: Direction: p value =	9291 : 9175 ↑ : ↓ 0. 996	8049 : 9175 ↓ : ↑ 0. 546
	SCFAs		
acetate	Median integral: Direction: p value =	73314 : 73408 ↓ : ↑ 0. 146	76475 : 73408 ↑ : ↓ 0. 898
butyrate	Median integral: Direction: p value =	83827 : 87358 ↓ : ↑ 0. 277	85135 : 87358 ↓ : ↑ 0. 145
	TCA metabolites and amino acids		
citrate	Median integral: Direction: p value =	444265 : 397881 ↑ : ↓ 0. 151	465199 : 397881 ↑ : ↓ **0.016**
succinate	Median integral: Direction: p value =	35874 : 33455 ↑ : ↓ 0. 425	33286 : 33455 ↓ : ↑ 0. 787
alanine	Median integral: Direction: p value =	112911 : 129213 ↓ : ↑ **0. 003**	113088 : 129213 ↓ : ↑ **0. 005**

Table 3.9a: Targeted univariate analysis using a Mann-Whitney U test of IBD subjects with no comorbidities and healthy controls. Figures in bold have a p value less than 0.05 after Benjamini-Hochburg correction for multiple comparisons. Arrows denote whether relative abundance of metabolite is higher or lower in corresponding cohort.

	All UC vs all controls (C), and all CD vs all UC		
n		UC : C *28 : 100*	CD : UC *74 : 28*
	Microbial metabolites		
4-cresol sulfate	Median integral: Direction: p value =	10141 : 14724 ↓ : ↑ **0.001**	12833 : 10141 ↑ : ↓ **0.015**
formate	Median integral: Direction: p value =	11618 : 12383 ↓ : ↑ 0.108	11295 : 11618 ↓ : ↑ 0.255
hippurate	Median integral: Direction: p value =	204281 : 283119 ↓ : ↑ 0. 079	190145 : 204281 ↓ : ↑ 0. 100
methylamine	Median integral: Direction: p value =	9198 : 9710 ↓ : ↑ 0. 645	9476 : 9198 ↑ : ↓ 0. 360
trigonelline	Median integral: Direction: p value =	10292 : 9175 ↑ : ↓ 0.601	8049 : 10292 ↓ : ↑ 0.515
	SCFAs		
acetate	Median integral: Direction: p value =	70314 : 73408 ↑ : ↓ **0. 010**	76475 : 70314 ↓ : ↑ **0.009**
butyrate	Median integral: Direction: p value =	82741 : 87358 ↓ : ↑ 0. 717	85135 : 82741 ↑ : ↓ 0. 219
	TCA metabolites and amino acids		
citrate	Median integral: Direction: p value =	432756 : 397881 ↑ : ↓ 0.823	465199 : 432756 ↑ : ↓ 0.083
succinate	Median integral: Direction: p value =	37814 : 33455 ↑ : ↓ 0. 073	33286 : 37814 ↓ : ↑ 0. 042
alanine	Median integral: Direction: p value =	111748 : 129213 ↓ : ↑ **0. 020**	113088 : 111748 ↑ : ↓ 0. 779

Table 3.9b: Targeted univariate analysis using a Mann-Whitney U test of IBD subjects with no comorbidities and healthy controls. Figures in bold have a p value less than 0.05 after Benjamini-Hochburg correction for multiple comparisons. Arrows denote whether relative abundance of metabolite is higher or lower in corresponding cohort.

Further comparisons of IBD and non-IBD cohorts were made with the inclusion and exclusion of non-IBD health conditions – this is summarised in Supplementary Tables 4 to 7. IBD groups including patients with comorbidities were compared against control groups that included subjects with non-IBD health conditions as well as healthy controls. Consistent changes in 4-cresol sulfate and hippurate were seen across comparison groups. Increased excretion of citrate was seen in IBD when subjects with comorbidities were included in the models. Reduced excretion of formate, alanine and acetate were seen in the UC cohorts when subjects with comorbidities were included.

In the last set of targeted analyses (row 4 in Supplementary Table 3, and Supplementary Table 6), IBD cohorts containing a comorbidity were compared against healthy controls. This showed changes in 4-cresol sulfate and hippurate seen in previous comparisons were preserved. Increased excretion of citrate was observed, a finding not seen in when subjects with comorbidities were excluded. Comparisons of IBD subjects with a comorbidity with non-IBD controls (that have at least one health condition) showed that in the UC cohort there was reduced excretion of 4-cresol, alanine and acetate.

The effect of comorbidities was assessed directly by comparing IBD cohorts with a comorbidity against IBD cohorts without comorbidities (row 4 in Supplementary Table 3, and Supplementary Tables 8 and 9) – an increase in 4-cresol sulfate and a reduction in acetate excretion was seen in IBD subjects with a comorbidity. Comparing healthy controls with non-IBD controls that have at least one health condition showed no differences in targeted metabolites.

3.6 Discussion

In contrast to previous urinary IBD metabonomic research, this study included all patients from IBD clinic except those taking [or had recently taken] antibiotics, and those with a stoma. This more open approach was adopted to try and include IBD subjects that reflected a more real-life clinic population, and help assess the impact of comorbidities on the IBD metabolic profiles, and to establish whether there is a potential diagnostic as well as research role of NMR metabolic profiling in a real-life IBD population.

The first part of this study assessed the effect of IBD on the faecal microbiome to help explain changes then observed in the urinary metabolome. This was performed in a subset of IBD patients with no comorbidities, and compared against healthy controls, in order to minimise the confounding effects of other conditions. It was then planned to assess and potentially correlate the changes on the IBD metabolome related to the presence of specific comorbidities such diabetes mellitus, but the number faecal samples in the IBD comorbid subjects were too small for meaningful analysis.

Consistent with previous studies (314-317), 16S RNA sequencing analysis showed a reduction in alpha diversity within the faecal microbiome when the Crohn's disease cohort was compared to healthy controls. No statistically significant changes could be seen when comparing UC to healthy controls, or the combined IBD group (Crohn's disease and UC) with controls. A reduction in alpha diversity measured using the Shannon index has been demonstrated in a recent study assessing microbiomic changes in UC patients compared to healthy controls (318), and bacterial richness in UC compared to controls was reported to be decreased in another study (319).

Compositional analysis showed changes in both Crohn's disease and UC groups when compared with healthy controls. There was an increase in *Bacteroidetes_unclassified* at class,

order and family level in the Crohn's disease cohort, and at order level in the UC cohort. Increased levels of *Bacteroidetes* phylum members in IBD have been reported in several studies (51, 52). At family level there was an increase in *Rumoninococcacea*, which is from the phylum Firmicutes. This family contains *Ruminococcus gnavus*; higher levels of this anaerobic bacteria have been observed in Crohn's disease which is thought to cause inflammation by loss of barrier function due its mucin-degrading capabilities (80, 320, 321).

At genus level, several changes were seen in both Crohn's disease and UC when compared to healthy controls. In the Crohn's disease cohort, a reduction in *Clostridia XIVb* and *XVIII* was seen. A lower abundance of *Clostridia* species has been reported in IBD, including *Clostridia XIVa*, which is important in maintaining gut health and is a SCFA producer (particularly butyrate) (75, 322). A reduction in *Blautia*, *Enterococcus*, *Selemonomnas*, *Holdermania*, *Haemophilus*, *Lactococcus* and *Desulfovibrionaceae_unclassified* was also seen in this study. In the UC cohort there was an increase in *Eggerthella*, which has been observed in previous studies, and is believed to be an emerging pathogen (322).

Correlating compositional microbiomic changes to urinary metabolic profiling in IBD, a reduction in hippurate and 4-cresol sulfate have been reported in previous studies (161, 207, 209, 210), and a positive correlation of these metabolites with *Clostridia* species and microbiomic diversity has been previously observed (323-325). Hippurate is a product of host-bacterial co-metabolism where dietary phenols are converted to benzoate by microbial action, and then undergoes hepatic conjugation by phase 2 glycine (326). 4-cresol sulfate is a product of bacterial metabolism of tyrosine, and along with *Clostridia* species, has a positive correlation with *Bacteroidetes* (324, 327).

This study analysed urinary samples from 315 patients, which included 117 Crohn's patients, 98 UC patients and 100 non-IBD controls, and included patients with comorbidities in both the

IBD groups and non-IBD groups. Subjects with stomas, those who had recently taken antibiotics, and those taking pre- and probiotics were excluded. Samples from 112 patients taking 5-aminosalicylates were analysed, which included 43 Crohn's disease patients taking this medication. The Crohn's disease patients taking 5-aminosalicyates (all Mesalazine) had predominantly colonic disease, or ileocolonic disease (27/43 and 9/43 respectively). Although this appears to be a high proportion of Crohn’s disease subjects taking 5-ASAS, these patients were recruited in 2015 and 2016 prior to the 2019 BSG guidelines where 5-ASAs are not recommended in Crohn’s disease (103) – of note, 75% of colonic Crohn’s disease patients in the population-based EPIMAD study were prescribed 5-ASAs (328).

Initial supervised multivariate analysis showed that separation between IBD cohorts and controls were principally driven by the presence of 5-aminosalicylates. This has been observed in previous studies (161, 211, 215) which have shown high concentrations of 5-ASAs excreted in the urine leading to intense NMR spectral resonances in the aromatic region. Many IBD patients (mainly UC) take 5-ASAs, and so excluding these patients from urinary NMR analysis removes an important cohort for ongoing metabonomic research, and reduces its potential role in clinical practice. Removing the aromatic region of the NMR spectrum results in the loss of a vital part of the NMR spectrum where several microbial associated metabolites are expressed. This study employed statistical total correlation spectroscopy editing (STOCSY-E) to recover the underlying spectral information lost due to the presence of 5-aminosalicylates in the aromatic region. Unfortunately, correlation and scaling of the 5-ASA peaks could not be achieved due to significant peak overlapping. As such the intestinal bacterial associated metabolites could not be recovered in these subjects, and so all IBD patients taking 5-aminosalicylates had to be removed from further analysis. This significantly impacted on samples sizes, particularly in the UC cohorts which had a much higher proportion of patients taking 5-ASA medications. It also meant that comorbidities then had to be grouped together, as

cohorts of patients with specific comorbidities (e.g. diabetes mellitus) were too small to analyse individually. This then meant that impact of metabonomic profiles specific to each different comorbidity on the IBD metabolome could not be assessed.

Subsequent analysis included 74 Crohn’s disease, 28 with ulcerative colitis, and 100 controls subjects. Metadata analysis showed there were several characteristics that were statistically different between different cohort comparisons (Table 3.6). In the IBD and Crohn's disease cohorts versus controls models this included age, ethnicity, and BMI. Age and BMI have been shown to effect over 180 urinary metabolites (329), including several related to the intestinal microbiome: dimethylamine, phenylacetylglutamine, 4-cresol sulfate, and hippurate (267).

Characteristic comparisons between the groups also showed differences in the total number of non-IBD health conditions between the IBD and Crohn’s disease cohorts when compared to controls, and specifically the presence of type 2 diabetes mellitus. Type 2 diabetes mellitus has been associated with changes in intestinal associated metabolites including hippurate, trimethylamine N-oxide, and dimethylamine (224, 225).

Lifestyle comparisons between groups in this study showed several differences (Table 3.7), but one of the most significant dietary influences reported in the literature has been a lactovegatarian diet (330). Although a significant difference in vegetarians relative to omnivores was not seen in this study, consumption of meat within the 24 hours prior to be providing a urine sample differed in the Crohn’s disease cohort compared to controls. Differences in urinary metabolites observed in lactovegetarians reported in one study (330) included an increase in excretion of hippurate, succinate and citrate, and decreased excretion of formate. A higher concentration of citrate was observed in the Crohn’s disease cohort compared to controls (Table 3.12), but this is unlikely to be of biological significance as this

finding was also seen in other cohort comparison where there was no significant difference in meat consumption.

In this study, multivariate analysis between combined IBD and controls, which included subjects with comorbidities, showed reduced excretion of hippurate, alanine, citrate, formate, p-cresol and dimethylglycine, and increased excretion of phenyacetylglutamine (PAG). These changes were present in the Crohn's disease versus control comparison, and present when comparing combined IBD and Crohn's disease cohorts with controls when all subjects with comorbidities have been removed. The reduction in hippurate and 4-cresol sulfate concentration in the IBD and Crohn's disease cohorts may be related to the reduction in *Clostridia* species seen in the 16S rRNA sequencing of the faecal samples.

Phenylacetylglutamine (PAG) excretion is produced almost exclusively from bacterial phenylalanine metabolism (331), and has been related to several bacteria including *Ruminococcaceae*, *Bifidobacteriaceae*, and *Coriobacteriaceae* (332, 333). This study showed an increase in *Ruminococcaceae* family in the Crohn's disease cohort when compared to controls and an increase in urinary phenylacetylglutamine. Phenylacetylglutamine also has an inverse relationship with BMI (267, 270), and both IBD and Crohn's disease groups in these comparisons had a lower BMI than the control group (see table 3.5 and 3.6). Therefore, higher levels of PAG may be a result of the lower median BMI in the IBD and Crohn's disease cohorts.

Lower excretion of dimethylglycine (DMG) was also shown to be discriminatory between IBD and Crohn's disease and controls in this study, which has not previously been reported. DMG is a produced by gut microbial degradation of choline, and has been shown to have a direct association with BMI (267). This finding may also be related to lower BMI in the IBD and Crohn's disease groups.

A lower concentration of the amino acid betaine was demonstrated in IBD and Crohn's disease when compared to controls on multivariate analysis, and this has been demonstrated previously associated with IBD (207).

Targeted analysis focused on ten metabolites that have been shown to be influenced by IBD, five of which are microbial related (4-cresol sulfate, formate, hippurate, methylamine, and trigonelline), two are SCFAs (acetate and butyrate), two are TCA metabolites, and one is amino acid (alanine). Reduced excretion of 4-cresol sulfate was the most consistent change across all IBD cohorts compared to non-IBD controls irrespective of the presence of comorbidities, and has been observed in a previous IBD study (161). 4-cresol sulfate has an inverse association with adiposity (267), but this did not appear to influence the lower concentration of this metabolite in the IBD cohorts which had a lower BMI.

Reduced hippurate excretion was demonstrated in targeted analysis of IBD and Crohn's disease cohorts irrespective of the presence of comorbidities (Table 3.12) but not in the UC against controls assessments – this might be due to the low number of UC participants in these comparisons. Hippurate has been consistently shown in previous studies to be of lower concentration in IBD compared to relative to controls (156).

This study showed a reduced concentration of alanine compared to controls in IBD and Crohn's disease, although this was only seen in cohorts where subjects with comorbidities were included. BMI and type 2 diabetes mellitus have a positive correlation with alanine, and so would not explain this finding.

Increased excretion of citrate was seen in the IBD and Crohn's disease groups where subjects with comorbidities were included relative to non-IBD controls. These IBD groups contained a significantly higher number of diabetic patients relative to controls (Table 3.6). Citrate concentrations have been observed to be increased with increasing glycosuria (225), and this

could explain the presence of this metabolite in the IBD and Crohn's disease cohorts compared to controls.

IBD cohorts with comorbidities and without comorbidities were compared against each other (Table 3.12, row 4). This showed no significant differences in targeted metabolites between these groups, and no differences in these metabolites were observed when comparing non-IBD subjects with health conditions compared to healthy controls.

This study has been limited by the effect of 5-aminosalicylates on the NMR spectrum, which significantly impacted on the number of UC subjects in this study. Comparison groups were reduced, which has likely affected the sensitivity of both multivariate analysis and targeted analysis. It has also led to potential confounding within the comparison groups with differences in age, BMI, ethnicity and the presence of comorbidities – differences in BMI and the presence of type 2 diabetes mellitus may have affected the concentrations of PAG and citrate respectively. Additionally, with lower subject numbers, comorbid IBD patients could not be stratified into specific groups for more in-depth analysis of specific comorbidities on the IBD metabolome.

Addressing the issue with 5-ASAs on the NMR spectrum may be difficult, and although this technique can be utilised around the time of diagnosis prior to 5-ASA medication being commenced, IBD patients already established on this treatment will likely require a more sensitive analytical platform such as gas chromatography mass-spectrometry (160).

Analysing all subjects' stool for microbiomic changes, rather than restricting this to non-comorbid subjects, would potentially have given additional information regarding the influence of a real-life population on the corresponding urinary metabolome.

Indirect correlation of microbiomic changes with changes in the urinary metabolome was performed in this study. To take this further, direct statistical correlation can be performed using Spearman's correlation tests, and Heatmap correlograms can then be produced to demonstrate bacterial and metabolite relationships which discriminate IBD from controls.

Overall, this study showed that changes in IBD associated metabolites, including microbial associated metabolites, are present when applied to a real-life population of patients with comorbidities, and changes in these metabolites can be partly linked to changes seen in the faecal microbiome. A larger study utilising a more sensitive analytical platform, and with subjects stratified into difference important comorbidities including type 2 diabetes mellitus, cardiovascular disease and inflammatory conditions, needs to be conducted to further assess the application of urinary metabonomics in a real-life population of IBD patients.

Chapter 4: Effects of obesity on the IBD urinary metabolome.

4.1 Summary

Obesity is a growing epidemic (245) and is affecting patients with IBD as much as the general population (243), and the pro-inflammatory properties of adipose tissue may influence the clinical course of this IBD. Changes in the intestinal microbiome and metabolome has been observed in obesity, but the influence of obesity on the urinary IBD associated metabolome has not been investigated. This study assessed the influence of obesity on discriminatory metabolites previously observed in IBD in 36 obese IBD patients, 35 obese control subjects, 95 normal weight IBD patients, and 38 normal weight controls.

Unsupervised multivariate modelling demonstrated no clear clustering of obese subjects compared to those of normal weight subjects. Targeted analysis comparing obese with normal weight subjects in IBD and non-IBD cohorts showed an increase in dimethylamine excretion, a gut microbiota-derived metabolite that has been previously shown to be increased in obesity. A reduction in hippurate excretion, the most consistently reported discriminatory metabolite in IBD, was not observed in obese IBD patients relative to obese controls.

Overall, obesity may influence the IBD associated metabolome particularly a reduction of hippurate excretion was not seen in obese IBD relative to controls, and so a larger study is needed to further investigate the influence of obesity on the urinary metabolome to further investigate this.

4.2 Introduction

The prevalence of obesity in IBD is at least as common as it is in the general population (243), with 15–40% of patients with IBD being reported as obese (334). Nearly 60% of adults are now overweight, having almost doubled since 1980 in developed countries, and along with

rising obesity, the incidence and prevalence of IBD has been increasing with approximately 0.5% of adults in the Western world suffering with this disease (18).

The impact of obesity was assessed in its own separate chapter within this thesis in light of the pro-inflammatory state related to obesity, and its significant growing prevalence both in the general population and within IBD population.

Worse clinical outcomes have been associated with obesity in several autoimmune diseases (335), but there has been less consistency from published studies that obesity adversely affects IBD. A study by Seminerio et al. (336) reported a higher frequency of elevated CRP and lower IBD-related quality of life in obese subjects, and Hass et al. (20) observed a shorter time to first surgery in overweight relative to underweight IBD patients. Blain et al. (21) observed a shorter time to developing peri-anal complications. However, no differences in risk or number of IBD-related surgeries, IBD-related hospitalisations, and initiation of anti-TNF treatment have been also reported (17, 19, 20). Two studies have observed better clinical course outcomes in obesity, with Flores et al. (17) reporting lower rates of surgery, hospitalisation and treatment escalation to anti-TNF therapy, and Pringle et al. (22) reporting a lower prevalence of penetrating disease in obesity.

Obesity may influence the pathogenesis of IBD, with excessive adipose tissue in subcutaneous and visceral compartments recognised as perpetuating a chronic low-grade inflammatory state (23). Increased circulating pro-inflammatory cytokines, and altered interactions of cytokines and adipokines are associated with obesity (24). Mesenteric hyperplasia correlating with inflamed bowel in mainly Crohn's disease - fat wrapping - is thought to be associated with more immunological activity than other visceral adipose tissue (25, 337). Severity of Crohn's disease has also been observed to correlate with adipokine secretion (338) and adipocyte activation has been associated with increased bacterial translocation. Dysbiosis has been linked

to the pathogenesis of obesity, with a twins study by Turnbaugh et al. (339) demonstrating a reduction in bacterial diversity, and compositional changes including enrichment of *Actinobacteria* and lower *Bacteroidetes*. Intestinal microbial changes have been reported following bariatric surgery including a reduction in *Firmicutes* encompasses *Clostridia* species (340, 341), correlating with reduced short chain fatty acids and bile acids in blood and faeces (342, 343).

Studies assessing the effect of obesity on the urinary metabolome have reported differences in intestinal microbial associated metabolites including an inverse relationship with hippurate, formate, and phenylacetylglutamine (PAG), and a correlation with dimethylamine and 4-cresol sulfate (267, 270) – all these metabolites with the exception of PAG and dimethylamine have been associated with IBD (161, 207, 209, 210).

Obesity has been defined in this study as a body mass index of greater than or equal to 30 kg/m^2. Issues around defining obesity based on BMI alone have been discussed previously in Chapter 1 subsection 1.2.4.4.

Previous studies examining the urinary metabolome in IBD have not accounted for the potential confounding of obesity within their study populations. For IBD urinary metabonomics to be applied to a real-life population, the influence of obesity needs to be assessed in an IBD population.

4.3 Aims and hypothesis

This chapter examines the effects of obesity on the urinary metabolome of IBD subjects, and specifically its effect on urinary metabolites known to be discriminatory in IBD.

Hypothesis: Discriminatory metabolites in IBD are specific to this disease and will not be influenced by high body mass index (BMI).

4.4. Methods

The methodology in this chapter heavily overlaps that of the previous chapter, and so this section will concentrate on aspects specific to this study.

This study had ethical approval from Imperial College Healthcare NHS Trust Research and Ethics Committee (Ref: 13/LO/1867). IBD subjects were recruited from IBD clinics from Imperial College Healthcare NHS Trust (ICHT), at the St Mary's Hospital and The Hammersmith Hospital sites, and London North West University Hospitals NHS Trust, at the Ealing Hospital site. Non-IBD controls were recruited from subjects working at ICHT and Imperial College London (St Mary's Hospital site), and from bariatric clinics at the same site.

Detailed clinical data, dietary and medication information, obtained at the time of sample collection. Exclusion criteria included subjects with IBD unclassified, stomas, and currently pregnant. Subjects who had recently taken antibiotics (within 8 weeks), pre- or probiotics, or on liquid or treatment diets were also excluded.

Subject recruitment, sample collection, handling, and recruitment were all done as described in Chapter 3. Subject characteristic and metabonomic analysis – both multivariate and targeted univariate analysis – were performed as described in Chapter 3.

Obesity within both IBD and non-IBD control cohorts was defined as a body mass index (BMI) greater than or equal to 30 kg/m2, and normal weight was defined as subjects with a BMI of greater than or equal to 20 kg/m2, and upto a BMI of 24.9 kg/m2.

Eleven metabolites were selected for targeted univariate analysis – this included the 10 metabolites selected in Chapter 3 which have shown to be discriminatory in IBD with six also been observed to be influenced by BMI (hippurate, 4-cresol sulfate, formate, citrate, succinate

and alanine), and in addition dimethylamine was also selected; a microbial associated metabolite excreted in higher concentration in obese subjects (267).

Multivariate and targeted univariate analysis was performed as described in Section 3.4.6 in Chapter 3.

4.5 Results

4.5.1: Characteristics of study participants

71 subjects with a BMI >30 kg/m^2 were recruited into this study – 36 with IBD and 35 non-IBD controls. 133 subjects with a normal BMI measuring between 20 and 25 kg/m^2 were also included in this study. Table 4.1 details the study participant characteristics.

	High BMI		Normal BMI	
Cohort	IBD n = 36	Controls n = 35	IBD n = 95	Controls n = 38
BMI (median, kg/m^2)	34.7 (IQR 5.4)	32.0 (IQR 3.4)	22.4 (IQR 2.23)	22.2 (IQR 3.02)
CD : UC	22 : 14	-	53 : 42	-
Age (median, in years)	53.5 (IQR 19.0)	54.5 (IQR 12.0)	47.4 (IQR 26.0)	49.1 (IQR 22.5)
Sex (M : F)	16 : 26	23 : 12	44 : 51	16 : 22
Ethnicity (Cau : SA : Oth)*	23 : 12 : 7	15 : 14 : 6	59 : 28 : 8	14 : 7 : 1
T2DM (CD : UC)	7 (3 : 4)	10	8 (3 : 5)	2

Table 4.1: Participant characteristics.

Subject characteristics were examined to assess for significant differences between different cohort comparisons – see Table 4.2. This showed a significant difference in age between IBD obese subjects relative to normal weight IBD subjects. There was also a significant difference in the number of subjects with type 2 diabetes mellitus in non-IBD obese subjects relative to non-IBD normal weight subjects. Lifestyle comparisons were made between different IBD groups and non-IBD obese and normal weight groups – see Supplementary Table 10.

	IBD obese v normal weight p value	Non-IBD obese v normal weight p value	Obese IBD v obese controls p value	Normal weight IBD v normal weight controls p value
Age[1]	**0.023**	0.117	0.881	0.542
Sex[2]	0.050	0.715	0.016	0.659
Ethnicity[2]	0.114	0.222	0.525	0.494
T2DM[2]	0.233	**0.007**	0.209	0.436

Table 4.2 – Participant characteristic comparisons. [1]Mann-Whitney U test, [2]Chi-squared test, p value of <0.05 considered significant and are in bold.

4.5.2: Multivariate analysis

The following figure (Figure 4.1) is an PCA model comparing IBD patients with a high BMI (≥ 30 kg/m^2) against normal weight IBD patients (BMI 20.0 to 29.9 kg/m^2). This shows no subject clustering according to obesity, and subsequent supervised analysis using OPLS-DA modelling showed no significant differences between obese and normal weight subjects.

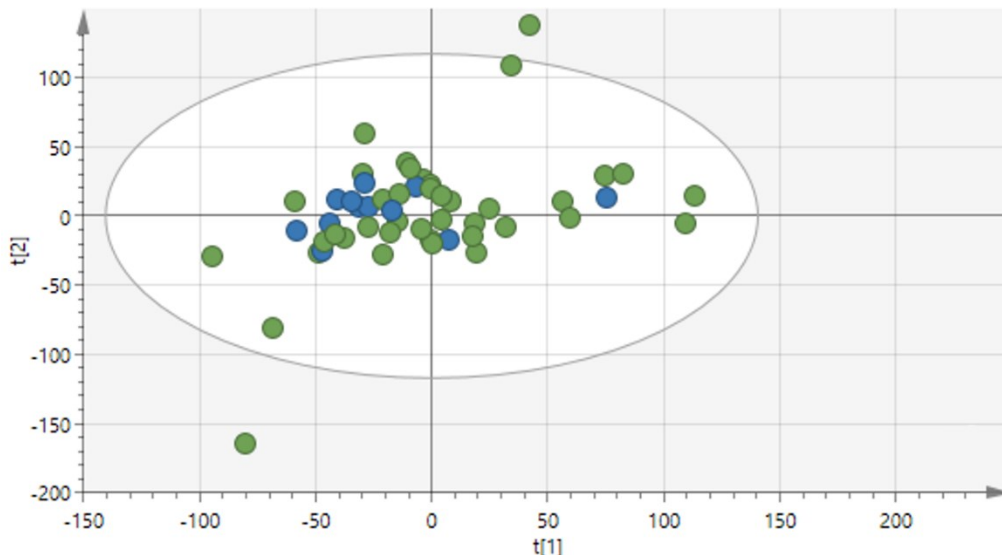

Figure 4.1: Unsupervised multivariate analysis (PCA) of IBD subjects with a high BMI (≥30 kg/m^2) compared with IBD subjects with a normal BMI (20-25 kg/m^2). Blue = high BMI cohort, green = normal weight.

4.5.3: Targeted analysis

Targeted univariate analysis of 11 targeted metabolites were assessed in IBD obese and normal weight cohorts. A significant reduction in dimethylamine excretion was seen in obese relative to normal weight subjects in both IBD (combined Crohn's disease and UC) and non-IBD cohorts, and UC only cohorts. A reduction in succinate was also observed in obese UC subjects compared to UC patients with a normal BMI. No significant differences were observed comparing obese Crohn's disease subjects when compared with normal weight Crohn's disease subjects.

	Obese vs normal weight (nw) in IBD, Crohn's disease (CD), ulcerative colitis (UC), and non-IBD cohorts				
n		IBD obese vs IBD normal weight (nw) *36 : 95*	CD obese vs CD nw *22 : 53*	UC obese vs UC nw *14 : 42*	Non-IBD obese vs non-IBD normal weight *35 : 38*
	Microbial metabolites				
4-cresol sulfate	Median integral:	10188 : 11961	12755 : 12916	9084 : 10974	14110 : 14220
	Direction:	↓ : ↑	↓ : ↑	↓ : ↑	↓ : ↑
	p value =	0.412	0.635	0.337	0.734
formate	Median integral:	11927 : 13580	12630 : 13951	11487 : 13111	13987 : 13400
	Direction:	↓ : ↑	↓ : ↑	↓ : ↑	↑ : ↓
	p value =	0.314	0.978	0.181	0.690
hippurate	Median integral:	378148 : 383904	436264 : 363944	341825 : 409091	467929 : 472679
	Direction:	↓ : ↑	↑ : ↓	↓ : ↑	↓ : ↑
	p value =	0.678	0.550	0.099	0.225
methylamine	Median integral:	9622 : 11287	10035 : 11484	9364 : 11038	9309 : 11216
	Direction:	↓ : ↑	↓ : ↑	↓ : ↑	↓ : ↑
	p value =	0.041	0.148	0.259	0.231
trigonelline	Median integral:	11863 : 14762	14926 : 13100	9949 : 16860	9156 : 14149
	Direction:	↓ : ↑	↑ : ↓	↓ : ↑	↓ : ↑
	p value =	0.393	0.439	0.035	0.134
dimethylamine	Median integral:	53940 : 51049	52235 : 51122	55005 : 50957	56869 : 54100
	Direction:	↑ : ↓	↑ : ↓	↑ : ↓	↑ : ↓
	p value =	**0.011**	0.497	**0.006**	**0.013**
	SCFAs				
acetate	Median integral:	83184 : 80841	77713 : 85157	86604 : 75396	88311 : 92092
	Direction:	↓ : ↑	↓ : ↑	↑ : ↓	↓ : ↑
	p value =	0.911	0.999	0.842	0.555
butyrate	Median integral:	91808 : 110019	87412 : 84770	94556 : 141881	88225 : 97140
	Direction:	↑ : ↓	↑ : ↓	↓ : ↑	↓ : ↑
	p value =	0.908	0.664	0.933	0.883
	TCA metabolites and amino acids				
citrate	Median integral:	426357 : 468813	370315 : 419108	461383 : 531535	521485 : 522726
	Direction:	↓ : ↑	↑ : ↓	↓ : ↑	↓ : ↑
	p value =	0.336	0.384	0.232	0.965
succinate	Median integral:	37270 : 40402	39237 : 37063	36040 : 44615	38630 : 42482
	Direction:	↓ : ↑	↑ : ↓	↑ : ↓	↓ : ↑
	p value =	0.103	0.957	**0.007**	0.242
alanine	Median integral:	149851 : 126563	128098 : 121949	163447 : 132387	164980 : 130722
	Direction:	↑ : ↓	↑ : ↓	↑ : ↓	↑ : ↓
	p value =	0.089	0.334	0.142	0.255

Table 4.3: Targeted univariate analysis comparing obesity cohorts against normal weight cohorts. A Mann-Whitney U test was used to generate a p value. Changes with statistical significance ($p<0.05$) after Benjamini-Hochburg correction are in bold. Obese subjects have BMI ≥ 30 kg/m^2, and normal weight have BMI between 20 and 25 kg/m^2.

Table 4.4 shows univariate analysis of IBD compared to non-IBD controls in obese and normal weight subjects. In obese subjects with IBD (combined Crohn's disease and UC), and obese UC subjects, there was a significant reduction in 4-cresol sulfate excretion when compared to obese controls. A reduction of 4-cresol sulfate excretion was seen in the normal weight IBD cohorts compared to controls, but was not significant after being tested for multiple comparisons.

Excretion of hippurate was lower in normal weight IBD subjects relative to controls, but in the obese IBD cohorts compared to obese controls this change was lost, with this being the most consistent discriminatory urine metabolite separating IBD from controls across previous studies.

	IBD vs controls in obese and normal weight cohorts				
n		Obese IBD v obese Controls *36 : 35*	Normal weight (nw) IBD v nw controls *95 : 38*	Obese CD v obese controls *22 : 35*	Obese UC v obese controls *14 : 35*
	Microbial metabolites				
4-cresol sulfate	Median integral: Direction: p value =	10188 : 14110 ↓ : ↑ **0.005**	11961 : 14220 ↑ : ↓ 0.023	12755 : 14110 ↓ : ↑ 0.150	9084 : 14110 ↓ : ↑ **0.006**
formate	Median integral: Direction: p value =	11927 : 13987 ↓ : ↑ 0.500	13580 : 13400 ↑ : ↓ 0.657	12630 : 13987 ↓ : ↑ 0.877	11487 : 13987 ↓ : ↑ 0.318
hippurate	Median integral: Direction: p value =	378148 : 467929 ↓ : ↑ 0.124	383904 : 472679 ↓ : ↑ **0.005**	436264 : 467929 ↓ : ↑ 0.685	341825 : 467929 ↓ : ↑ 0.082
methylamine	Median integral: Direction: p value =	9622 : 9309 ↑ : ↓ 0.623	11287 : 11216 ↑ : ↓ 0.737	10035 : 9309 ↑ : ↓ 0.313	9364 : 9309 ↑ : ↓ 0.456
trigonelline	Median integral: Direction: p value =	11863 : 9156 ↑ : ↓ 0.299	14762 : 14149 ↑ : ↓ 0.741	14926 : 9156 ↑ : ↓ 0.200	9949 : 9156 ↑ : ↓ 0.512
dimethylamine	Median integral: Direction: p value =	53940 : 56869 ↓ : ↑ 0.887	51049 : 54100 ↓ : ↑ 0.618	52235 : 56869 ↓ : ↑ 0.657	55005 : 56869 ↓ : ↑ 0.582
	SCFAs				
acetate	Median integral: Direction: p value =	83184 : 88311 ↓ : ↑ 0.275	80841 : 92092 ↓ : ↑ 0.422	77713 : 88311 ↓ : ↑ 0.938	86604 : 88311 ↓ : ↑ 0.346
butyrate	Median integral: Direction: p value =	91808 : 88225 ↑ : ↓ 0.484	110019 : 97140 ↑ : ↓ 0.373	87412 : 88311 ↓ : ↑ 0.439	94556 : 88311 ↑ : ↓ 0.999
	TCA metabolites and amino acids				
citrate	Median integral: Direction: p value =	426357 : 521485 ↓ : ↑ 0.128	468813 : 522726 ↓ : ↑ 0.431	370315 : 521485 ↓ : ↑ 0.027	461383 : 521485 ↓ : ↑ 0.484
succinate	Median integral: Direction: p value =	37270 : 38630 ↓ : ↑ 0.959	40402 : 42482 ↓ : ↑ 0.803	39237 : 38630 ↑ : ↓ 0.923	36040 : 38630 ↓ : ↑ 0.894
alanine	Median integral: Direction: p value =	149851 : 164980 ↓ : ↑ 0.160	126563 : 130722 ↓ : ↑ 0.105	128098 : 164980 ↓ : ↑ 0.023	163447 : 164980 ↓ : ↑ 0.522

Table 4.4: Targeted univariate analysis comparing IBD cohorts against controls in obese and normal weight subjects. A Mann-Whitney U test was used to generate a p value. Changes with statistical significance ($p<0.05$) after Benjamini-Hochburg correction are in bold. Obese subjects have BMI ≥ 30 kg/m^2, and normal weight have BMI between 20 and 25 kg/m^2.

4.6 Discussion

Obesity, a growing issue in the IBD population (243), is associated with a chronic low inflammatory state - along with alterations in pro-inflammatory cytokines, reduced barrier function and dysbiosis are also thought to be related. Changes in urinary intestinal microbial associated metabolites have been observed in obesity, with several metabolites also being discriminatory in IBD. This study assessed whether the presence obesity was significant in influencing the IBD associated urinary metabolome.

This study recruited relatively small numbers compared to the previous study, and this meant there was a lot of heterogeneity within the comparison groups, and this may well have impacted on the results – this was in part having to exclude subjects taking 5-aminosalicylates.

Unsupervised multivariate modelling demonstrated no clear clustering of obese subjects compared to those of normal weight subjects. This may suggest that obesity is relatively less important in the clustering of subjects compared to other factors such as IBD. However, the heterogeneity of the subjects within the model, and potentially the number of participants in this study may also explain the lack of clustering. Supervised multivariate analysis did not show differences between obese and non-obese subjects in this study.

Targeted analysis comparing obese with normal weight subjects in IBD and non-IBD cohorts showed an increase in dimethylamine excretion ($p = 0.011$ and 0.013 respectively), and this difference was mirrored in the UC obese cohort relative to normal weight UC patients ($p = 0.006$). No changes between obese and normal weight subjects were elicited in the Crohn's disease cohort – which may be due to the relatively low number of subjects within the comparison. A higher concentration of dimethylamine has been previously observed in obesity (267). Dimethylamine is converted from both trimethylamine N-oxide and trimethylamine, with trimethylamine liberated by gut microbes from dietary precursors including choline (344).

Examining obese IBD patients with obese controls demonstrated a reduction in 4-cresol sulfate in UC subjects, and differences in citrate and alanine in Crohn's disease subjects. No significant change in hippurate excretion was observed, one of the most consistently observed findings associated with IBD, but reduced excretion was observed in normal weight IBD subjects compared to controls. The obese IBD and non-IBD cohorts contained significantly more type 2 diabetic subjects compared to the normal weight cohorts, and with hippurate excretion having been reported as increased with increasing glycosuria and glycohaemoglobin, this might explain the non-significant change in hippurate concentration within the obese IBD cohort.

The main limitation of this study was the relatively low number of participants, which was affected in part by the exclusion of subjects taking 5-aminosalicylates which could not be overcome with STOCSY-E. With more heterogeneity in comparison groups caused by the inclusion of IBD and non-IBD subjects with other health condition (as in Chapter 3), smaller study participant numbers will be a more significant confounding factor.

Overall, although multivariate analysis showed no clustering of obese patients, obesity may influence the IBD associated urinary metabolome as no change in the excretion of hippurate was observed in obese IBD patients relative to obese controls. A larger study is needed to further assess the influence of obesity in IBD urinary ^{1}H NMR experiments.

Chapter 5: Examining the effect of bowel purgatives on the urinary metabolome

This chapter has been published as an original research paper:

Powles, S. T., et al. (2022). "Effects of bowel preparation on intestinal bacterial associated urine and faecal metabolites and the associated faecal microbiome." BMC gastroenterology 22(1): 1-9.

5.1 Summary

Urinary and faecal metabolic profiling has been extensively studied in GI diseases as potential diagnostic markers, and to enhance our understanding of the intestinal microbiome in the pathogenesis these conditions. The impact of bowel cleansing on the microbiome has been investigated in several studies, but limited to just one study on the faecal metabolome. The aim of this study was to compare the effects of bowel cleansing on the faecal microbiome, and the urine and faecal metabolome.

Urine and faecal samples were obtained from eleven patients undergoing colonoscopy at baseline, and then at day 3 and week 6 after colonoscopy. 16S rRNA gene sequencing was used to analyse changes in the microbiome; assessing changes in alpha and beta diversity, and specific taxonomic changes at phylum to genus levels. Metabolomic analysis was performed using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. Multivariate analysis assessed dissimilarity between samples at different time points, and targeted univariate analysis investigated the effects of bowel cleansing on 20 urine and 10 faecal bacterial associated metabolites.

Microbiomic analysis demonstrated a change in alpha diversity (Shannon index) between samples taken at baseline and 3 days following bowel cleansing ($p = 0.002$), and no significant change between samples at baseline and 6 weeks post colonoscopy. Beta analysis showed that

intra-subject variability was greater than inter-subject variability. There were no significant taxonomic changes between time points. Targeted and non-targeted analysis of urinary and faecal bacterial associated metabolites showed no significant impact following bowel cleansing.

In conclusion, bowel cleansing causes a temporary disturbance in bacterial alpha diversity measured in faeces, but no significant changes in the faecal and urine metabolic profile, suggesting that the faecal microbiome and its associated metabolome is resistant to the effects of an induced osmotic diarrhoea.

5.2 Introduction

Investigation of the composition and functionality of the gut microbiome is of key interest for a range of GI diseases. However, there is no standardised best practice regarding choice of sample type (stool or mucosal biopsies), or methodology regarding sample collection and/or processing. It is also recognised that a wide range of external factors may influence the results of analysis. One major example would be medications, with bowel purgatives such as polyethylene glycol (PEG) solutions, which are given as bowel cleansing prior to colonoscopy (274, 275), being of particular relevance when investigating GI disease.

PEG solutions cause a profound osmotic diarrhoea with a high-volume lavage rapidly passing through the gastro-intestinal tract which in turn alters the luminal contents including the microbiota and luminal metabolites (276, 277). Effects of bowel cleansing on the intestinal microbiome have been studied (276-282), both to assess whether it can directly cause dysbiosis, and to assess how these vary in health and disease (277).

Results have been inconsistent across different published studies; some have shown a significant reduction in bacterial load (281) and alpha diversity (276, 279, 280) when examining the faecal and colonic mucosal microbiota post purgatives, but this has not been universally demonstrated

(277, 278, 282). In those studies that demonstrate a dysbiosis however it appears that the composition recovers quickly (277, 280, 281) - likely within 14 days, but the exact timing of this restoration is unclear. Understanding the longevity of these microbial changes may be important in determining study protocols and interpreting results. Of further interest is determining if there are metabolically functional changes (measured in the metabolome) associated with these microbiome perturbations, beyond simply measuring composition. Only one study to date (277) has assessed the metabolic effects of bowel cleansing on faecal samples, and showed that bowel cleansing caused a change in the faecal metabolome immediately after bowel cleansing, but this had recovered at day 14 sampling.

While faecal metabolic data have been extensively researched in GI conditions (216-218, 345-347), sample collection can be unappealing for patients, and therefore be more difficult to obtain. Urine is a more readily acquired biological sample, and has been shown to demonstrate gut host-microbiota metabolic changes in GI pathology including inflammatory bowel disease (IBD) (161, 207, 209, 210, 213) and colorectal cancer (348-351). No studies have yet been published assessing the impact of bowel cleansing on bacterial associated urinary metabolites.

Previous studies have assessed stability of the microbiome following bowel cleansing at various different time points. This study chose baseline sampling at 3 days pre-colonoscopy, and then 3 days after colonoscopy as the impact of dietary changes should have settled at this time, and then a final sampling date at 6 weeks post colonoscopy to see if more lasting changes in the metabolome and microbiome were present – as a previous study (281) had shown changes in the colonic microbiota at 28 days post 2L of PEG when compared to baseline, this study used 6 weeks to see if changes continued beyond 28 days.

5.3 Aims and hypothesis

This study aimed to compare the effects of bowel cleansing on the faecal microbiome and urinary and faecal metabolome at baseline, day 3 and week 6 after colonoscopy.

Hypothesis: Bowel cleansing using polyethylene glycol will have a transient and temporary effect on urinary and faecal metabolome correlating with a temporary change in the faecal microbiome.

5.4 Method

5.4.1 Experimental design and subjects

This study had ethical approval from Imperial College Healthcare NHS Trust Research and Ethics Committee (Ref: 13/LO/1867). Eleven subjects were recruited from gastroenterology clinics at St. Mary's Hospital in London who were due to undergo a colonoscopy. Subjects were excluded if they had received antibiotics, further purgatives, acid suppressing or immunosuppressive medication within 2 months of sample collection. Written informed consent was obtained from all participants. Detailed dietary and lifestyle data was taken from each subject.

5.4.2 Sample collection

32 urine samples and 30 faecal samples were collected from 11 subjects at 3 different time points from the time of their colonoscopy – see Table 5.1. 9 of these subjects were male, and the mean age was 41 years. 10 of the subjects were Caucasian, and one was black. The mean BMI was 23.4 kg/m^2. Two subjects did not provide faecal samples at the last time point, and one of these subjects also did not provide a urine sample at the last time point – however all recruited subjects and all samples were included in the final analysis. Baseline (t0) samples were collected 3 days

prior to the procedure, and before a low residue diet or bowel purgatives were commenced. Further samples were collected 3 days post procedure (t1), and 6 weeks post procedure (t2). Samples were collected in sterile polypropylene containers and stored in a -80ºC freezer once received by the subject. MoviPrep® (Macrogol 3350, Sodium sulphate anhydrous, Sodium chloride, Potassium chloride, Ascorbic acid and Sodium ascorbate) was used as bowel preparation in all cases.

Sbj.	**Age**	**Sex**	**Ethnicity**	**BMI**	**Comorbidities**	**Medications**	**Colonoscopy report**	**Histology**
1	41	M	Caucasian	27.2	Ulcerative colitis	Pentasa	Patchy erythema distally	Within normal limits
2	63	M	Caucasian	25.6	Ulcerative colitis, type 2 diabetes mellitus	Metformin	UC mild activity in distal 20cm	Quiescent colitis in rectum
3	31	M	Caucasian	19.7	Nil	Nil	Normal	Nil taken
4	28	M	Caucasian	34.6	Nil	Nil	Normal	Within normal limits
5	24	M	Caucasian	18.5	Nil	Nil	Normal	Within normal limits
6	45	F	Caucasian	21.3	Nil	Nil	Normal	Nil taken
7	47	M	Caucasian	23.6	Hypertension	Candesartan	Normal	Nil taken
8	31	M	Black	23.3	Nil	Nil	Normal	Nil taken
9	40	F	Caucasian	20.7	Nil	Nil	Normal	Within normal limits
10	38	M	Caucasian	20.1	Nil	Nil	Normal	Nil taken
11	58	M	Caucasian	22.6	Nil	Nil	Normal	Nil taken

Table 5.1: Characteristics of study subjects.

5.4.3 Bacterial DNA extraction and 16S rRNA gene sequencing

DNA extraction was performed using the PowerLyzer PowerSoil DNA Isolation Kit (Mo Bio, Carlsbad, CA, USA). 250 mg of faeces from each sample was used for extraction, and the manufacturer's instructions were followed. An in-house additional bead beating step (90) was included at speed 8 for 3 minutes using a Bullet Blender Storm (Chembio Ltd, St Albans, UK). The extracted DNA was then stored at -80ºC. Illumina's 16S Metagenomic Sequencing Library Preparation Protocol (289) was used to prepare the sample libraries.

16S rRNA sequencing data was then analysed using the Mothur package following the MiSeq SOP Pipeline (290). Sequence alignment was performed using the Silva bacterial database, and the Wang method using the RDP database was used for classification of sequences (292). Analysis for alpha and beta diversity was performed using the same method detailed in Chapter 3. The Statistical Analysis of Metagenomic Profiles software (STAMP) package (312) was used to assess for statistically significant differences in bacterial composition between subjects at different time groups.

5.4.4 Metabonomic analysis

One dimensional spectra were obtained from urine and faecal samples using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy.

Multivariate statistical analysis was used to investigate differences between study groups. This performed using SIMCA (version 15, Umetrics, Sweden). Principal components analysis (PCA) was carried out using univariate scaling to allow for the identification of any outliers and initial clustering based on principle components.

For targeted analysis, peak integral values for selected metabolites were obtained using an in-house Matlab script (90). Using the integral values, a univariate statistical approach was used to

compare the relative concentration of metabolites of interest between the three time points. GraphPad Prism statistical analysis software programme version 8.0.2 was used to perform a Wilcoxon matched-pairs signed rank test between time points t0 and t1, t0 and t2, and t1 and t2. A Bonferroni calculation was used to correct for multiple comparisons. Urine metabolites (161, 207, 209, 210, 212, 231, 348-351) and faecal metabolites (216-220) were selected for targeted analysis if they are produced by intestinal bacterial metabolism or host-bacterial co-metabolism, and in previous studies have been shown to be important in GI disease.

5.5 Results

5.5.1 Faecal 16s RNA Sequencing

31 faecal samples were collected, which included 11 samples at baseline (t0), 11 at three days post colonoscopy (t1), and 9 at six weeks post procedure (t2). Analysis of the alpha diversity between baseline (t0) and 3 days post procedure (t1) showed that bowel preparation caused a significant decrease ($p = 0.002$) in the Shannon index of the bacteria present (Figure 5.1). There was no significant change between baseline and 6 weeks post bowel cleansing (t0 and t2).

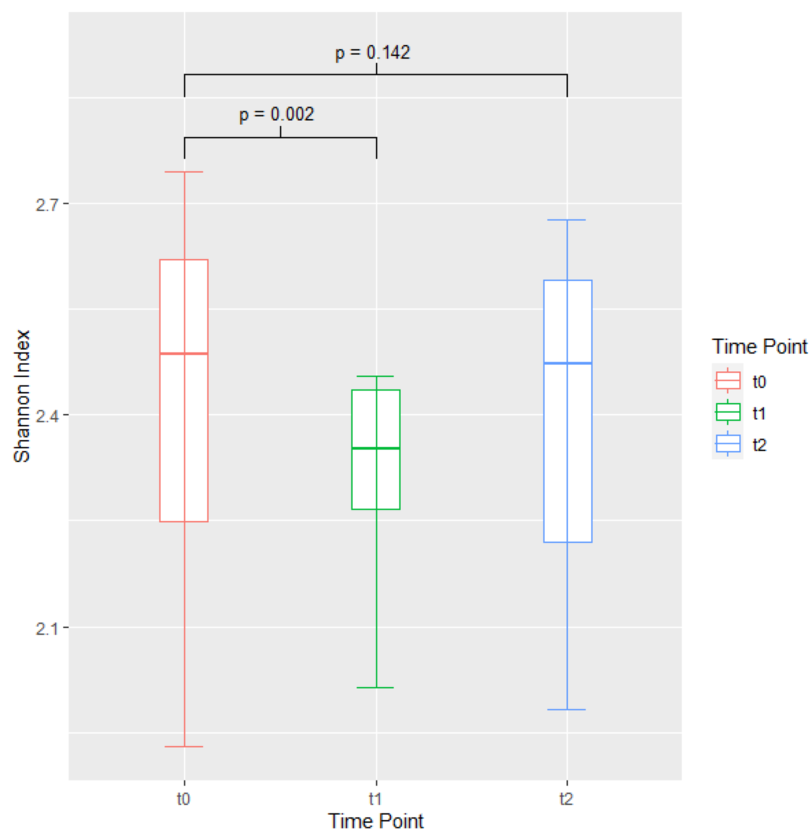

Figure 5.1: Faecal microbiota alpha diversity as measured by the Shannon index at baseline (t0), 3 days after bowel preparation (t1), and 6 weeks after bowel preparation (t2). Wilcoxon matched-pairs signed rank test (two-tailed) between t0 and t1 had a p value of 0.002, and between t0 and t2 there was a p value of 0.142.

Compositional changes between samples (beta diversity) was analysed using non-metric multidimensional scaling (NMDS) plots measuring weighted Unifrac distances. Weighted Unifrac distances compared the inter- and intra-subject variability between samples following the use of bowel preparation (Figure 5.2). Eight of the subjects had relatively conserved beta diversity despite the use of bowel preparation, and so in these cases the inter-subject variability was greater. The remaining three subjects had a considerable change in composition over the 3 time points (coloured in blue, orange and yellow), and in each of these cases there was a marked difference between baseline and 3 days following bowel cleansing.

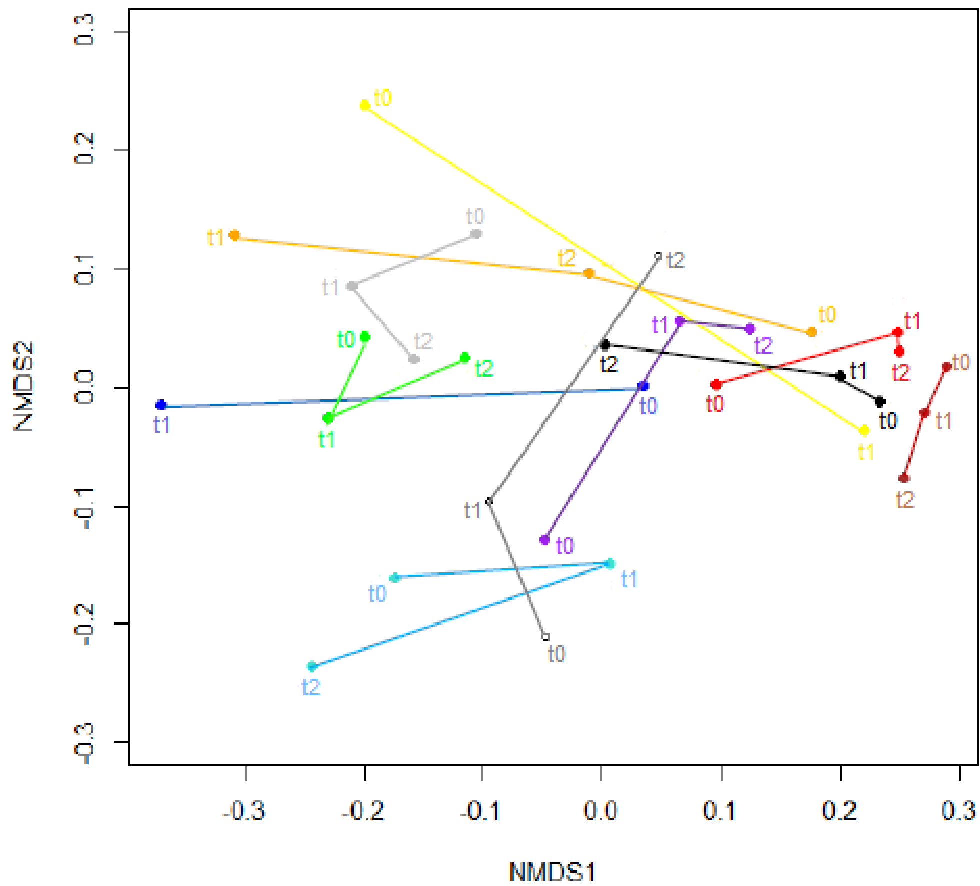

Figure 5.2: NMDS plot of microbial composition of subjects at differing time points. t0 = baseline, t1 = 3 days post colonoscopy, and t2 = 6 weeks post colonoscopy.

Taxanomic analysis at phylum, class, order, family and genus levels was performed using the STAMP software package, and showed no significant differences in composition of bacteria between samples taken at baseline, 3 days post colonoscopy, and 6 weeks post procedure (Figure 5.3).

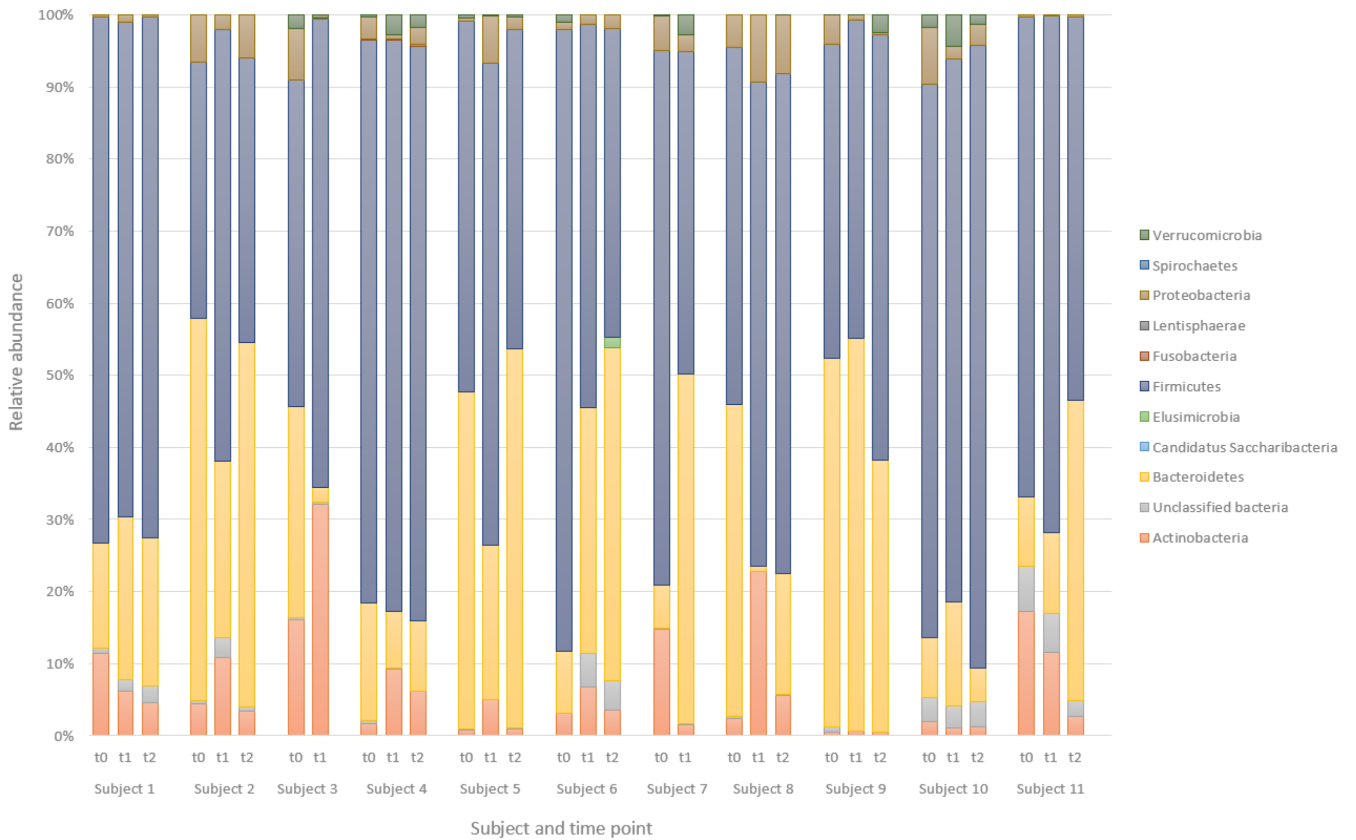

Figure 5.3: Percentage bar graph showing the relative proportion of bacterial phyla in each sample. Samples are grouped within subjects and in order of collection time; at baseline (t0), 3 days after bowel preparation (t1), and 6 weeks after bowel preparation (t2).

5.5.2 Urine metabonomic analysis

Metabonomic analysis was performed on 32 urine samples which were collected at the same time as the faecal samples. This included 11 baseline samples (t0), 11 collected at 3 days post procedure (t1), and 10 collected at 6 weeks post procedure (t2).

Unsupervised multivariate analysis using Principal Component Analysis (PCA) of the urine samples at each time point was performed – see Figure 5.4. This showed that inter-subject variability was greater than intra-subject variability irrespective of bowel preparation in 9 out of 11 subjects.

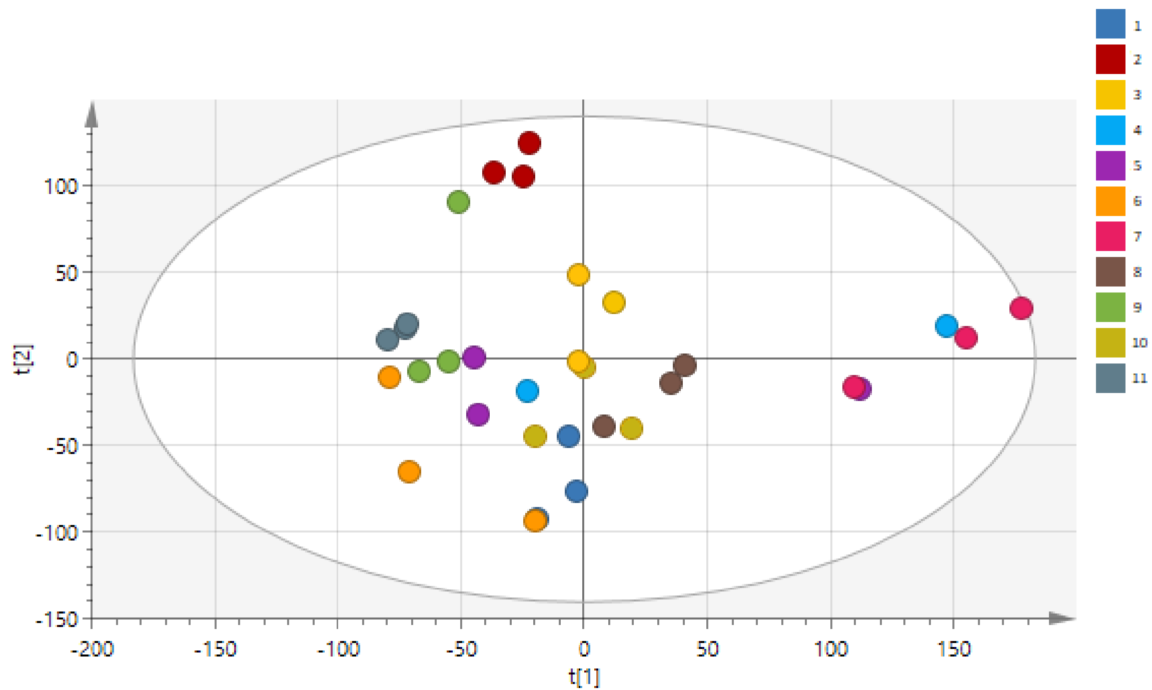

Figure 5.4: Unsupervised multivariate analysis of urine metabolites at each time point. PCA scores plot of urine metabolic profiles of 11 subjects, with samples at baseline, 3 days post procedure, and 6 weeks post procedure. Samples from the same subject are plotted in the same colour. Lines have been added to this figure to link time points between samples from the same subject.

Targeted analysis of 38 urine metabolites showed no significant change between time points t0 and t1 when corrected for multiple comparisons ($p < 0.003$) – see Figure 5.5 and Table 5.2.

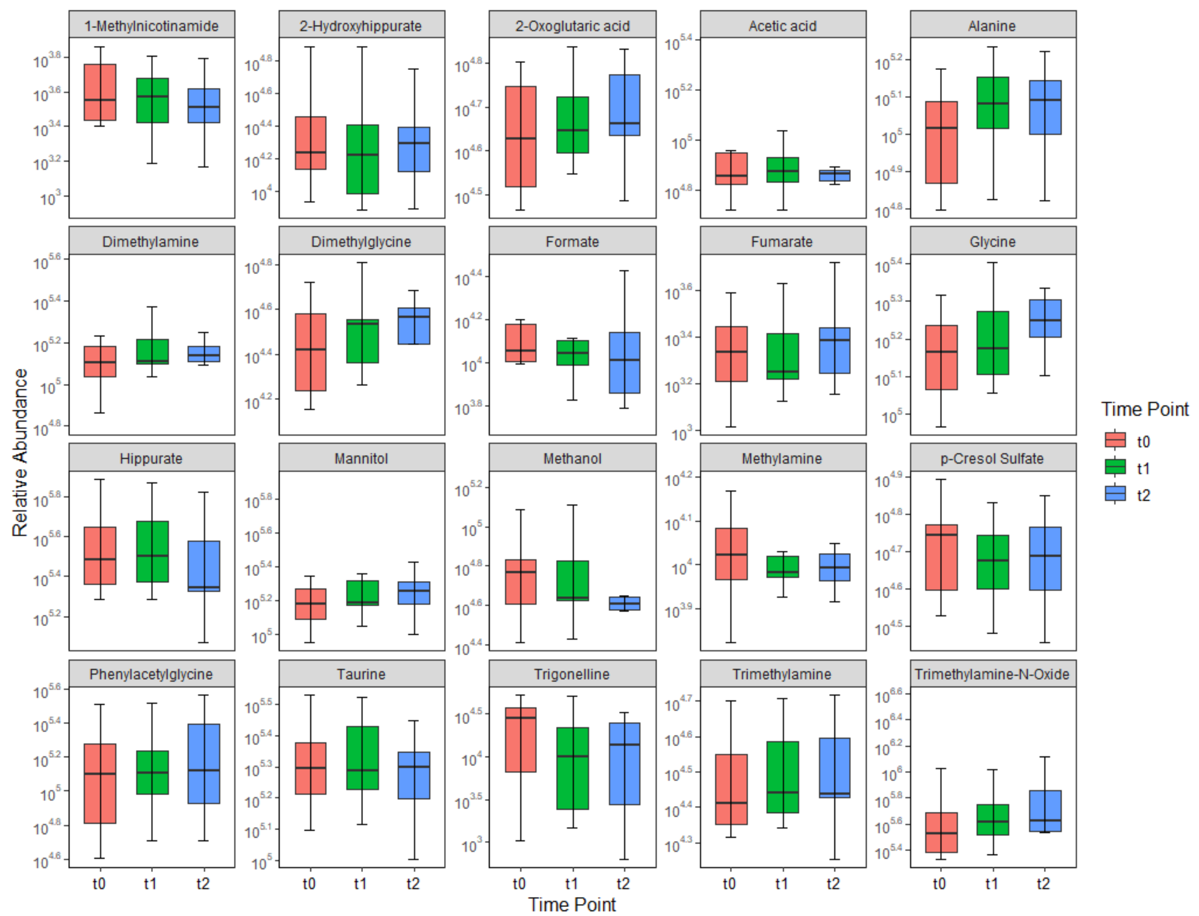

Figure 5.5: Bar plot graphs showing median relative quantity of each urine metabolite at three time points; at baseline (t0), 3 days after bowel preparation (t1), and 6 weeks after bowel preparation (t2).

Metabolite	p value		
	t0 vs t1	**t0 vs t2**	**t1 vs t2**
1-methylnicotinamide	0.577	0.057	0.492
2-hydroxyhippurate	0.240	0.846	0.923
2-oxoglutaric acid	0.320	0.492	0.432
acetic acid	0.465	0.492	0.929
alanine	0.005	0.020	0.770
4-cresol sulfate	0.365	0.846	0.922
dimethylamine	0.831	0.695	0.625
dimethylglycine	0.240	0.625	0.922
formate	0.765	0.322	0.695
fumurate	0.700	0.275	0.922
glycine	0.175	0.131	0.492
hippurate	0.966	0.770	0.557
mannitol	0.413	0.557	0.696
methanol	0.765	0.322	0.695
methylamine	0.520	0.770	0.375
phenyacetylglutamine (PAG)	0.638	0.322	0.492
timethylamine	0.831	0.432	0.770
taurine	0.700	0.625	0.769
trigonelline	0.413	0.193	0.432
trimethylamine N-oxide (TMAO)	0.898	0.625	0.846

Table 5.2: Univariate analysis comparing urine metabolites between time points. Changes in urine metabolites between baseline (t0), day 3 post bowel preparation (t1), and 6 weeks post procedure (t2). Wilcoxon matched-pairs signed rank test to assess for statistical significant ($p < 0.003$ after correction for multiple comparisons).

5.5.3 Faecal metabonomic analysis

Metabonomic analysis was performed on ten faecal samples at baseline (t0), and ten samples 3 days post colonoscopy (t1). Unsupervised multivariate analysis of faecal water metabolities was performed using Principal Component Analysis – see Figure 5.6. This showed that in 9 out of 10 subjects, the inter-subject variability was greater than the inter-subject variability. Targeted analysis was performed on 10 metabolites. This showed there were no significant differences after correction for multiple comparisons – see Figure 5.7 and Table 5.3.

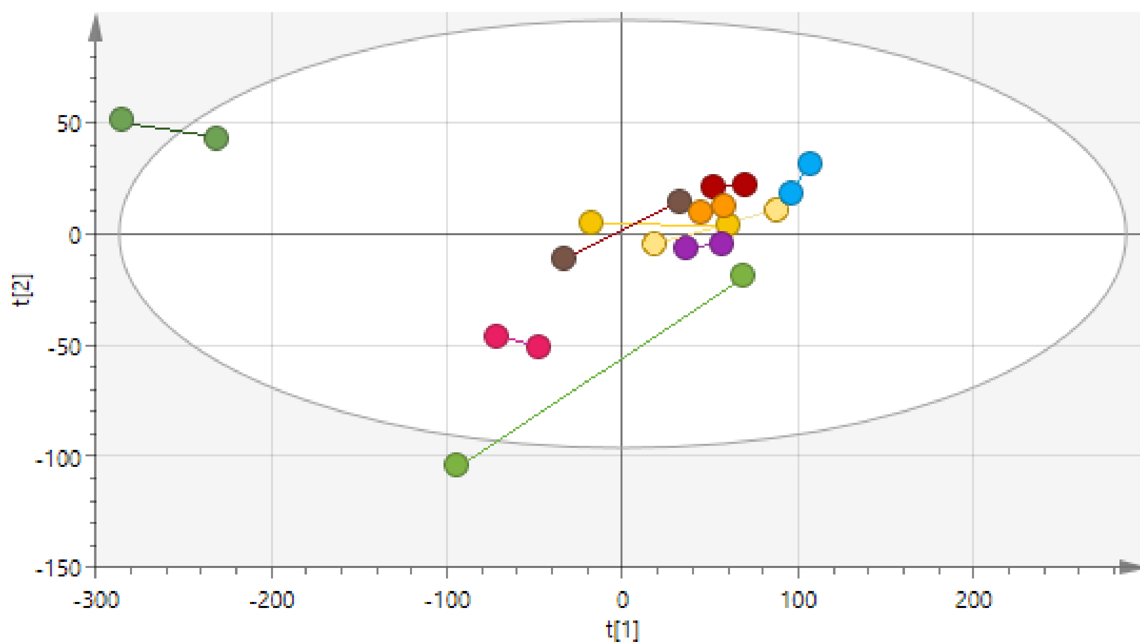

Figure 5.6: Unsupervised multivariate analysis of faecal water metabolites at each time point. PCA scores plot of faecal metabolic profiles of 11 subjects, with samples at baseline and 3 days post procedure. Samples from the same subject are plotted in the same colour. Lines have been added to this figure to link time points between samples from the same subject.

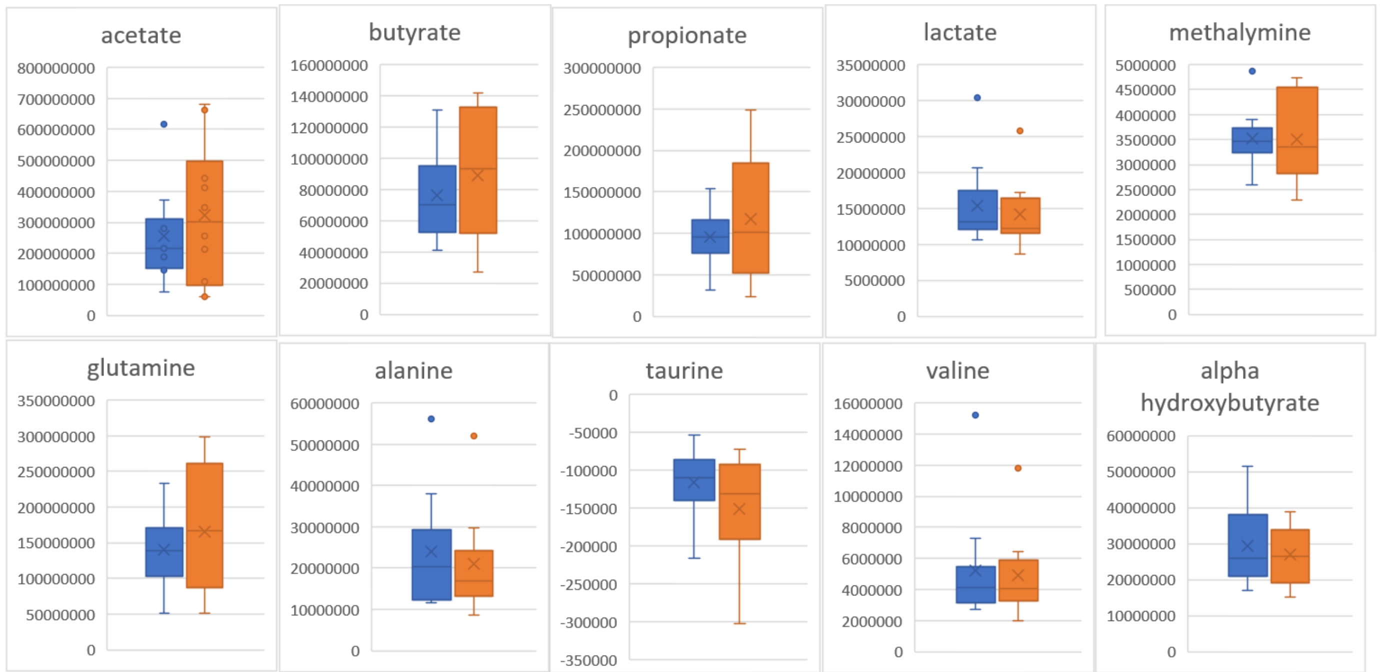

Figure 5.7: Bar plot graphs showing median relative quantity of each faecal metabolite at two time points; at baseline (t0), and 3 days after bowel preparation (t1).

Metabolite	p value - t0 vs t1*
acetate	0.084
butyrate	0.027
propionate	0.232
lactate	0.193
methylamine	0.695
glutamine	0.065
alanine	0.557
taurine	0.193
valine	0.846
alpha hydroxybutyrate	0.695

Table 5.3: Univariate analysis comparing faecal water metabolites between time points. Changes in faecal water metabolites between baseline (t0), day 3 post bowel preparation (t1), and 6 weeks post procedure (t2). Wilcoxon matched-pairs signed rank test to assess for statistical significant ($p < 0.005$ after correction for multiple comparisons).

5.6 Discussion

This is a novel study combining urinary and stool metabolic data with microbiomic changes to assess the effect of bowel cleansing pre-colonoscopy.

Stool analysis showed a reduction in microbiome ecological indices, with a significant decrease in the Shannon index ($p = 0.002$) following bowel preparation. Gorkiewicz et al. (276) demonstrated a reduction in richness following three days of PEG administration, and this change trended towards recovery but remained significantly lower than baseline one week after bowel cleansing. A trend towards reduced richness and Shannon index was shown by Shobar et al. (280) within one week post bowel cleansing in eight patients with IBD and ten healthy controls.

However, four other studies did not show a change in Shannon index following bowel cleansing (277, 278, 281, 282).

Subject clustering was more marked than time point clustering in all but three patients following bowel cleansing, and there were no statistically significant changes in beta diversity between samples across time points. Nagata et al. (277) observed using a principal coordinated analysis (PCoA) plot of weighted UniFrac distances that individuals who had received bowel cleansing clustered together, rather than clustering at time points. A study by Shobar et al. (280) also did not show a statistically significant difference in weighted Unifrac distances in faecal samples pre- and post-bowel cleansing, but did show a difference in unweighted distances, potentially suggesting that rarer species were affected more by bowel cleansing.

Taxonomic composition analysis showed no statistically significant changes from phylum to genus level following bowel cleansing. Nagata et al. (277) showed no differences at phyla level in eight patients receiving bowel cleansing, and in genera with a >1% relative abundance. A larger study by O'Brien et al. (282) showed no consistent findings in taxonomic composition in 15 patients undergoing bowel lavage. Another study by Shobar et al. (280) showed a reduction in *Bacteroidetes* in 10 healthy controls and 8 IBD patients receiving bowel preparation, along with changes at other taxonomic levels including a reduction in the *Clostridiales* order. A reduction in *Firmictues* and in an increase in *Proteobacteria* was reported in a study by Drago et al. (278) at phyla level, who also showed a reduction in the *Clostridia* class. Discrepancies in the literature in the previous studies (276-278, 280-283) to date assessing the impact of bowel cleansing on the faecal microbiota are likely in part due to the differing study designs, time point analysis, analytical techniques and generally small sample sizes.

The effects of bowel cleansing on intestinal bacterial associated urine metabolites was measured in this study, and the selected targeted metabolites which have been previously demonstrated to

alter in GI diseases including IBD and colorectal cancer (161, 207, 209, 210, 212, 231, 348, 349, 351). These included formate, hippurate, 4-cresol sulfate, and alanine (161, 209). Formate, produced both endogenously and also from intestinal fermentation, and has been associated with *Enterobacteriaceae* phylum, and particularly *Escherichia coli* (352, 353). Hippurate, a product of host and commensal co-metabolism of dietary aromatic compounds, has been shown in several studies to have reduced excretion in IBD (161, 207, 209, 210), and to have a positive association with *Clostridia* species (323). Bacterial fermentation of tyrosine in the colon produces p-cresol sulfate, and its production has also been associated with *Clostridia* species (324). Beta-alanine, an isomer alanine, is a non-protein amino acid obtained from dietary muscle protein and additionally sourced from intestinal *Escherichia coli* (354). Trimethylamine N-oxide (TMAO) has been associated with development of colorectal cancer (347, 355). TMAO is reduced to trimethylamine (TMA) by predominantly Enterobacteriaceae in the gut (356).

There were no significant changes in any of the urinary metabolites that were measured following bowel cleansing (with exception of alanine, although this was not significant when corrected for multiple comparisons). Unsupervised multivariate analysis showed that in nine out of eleven participants subject clustering was preserved following bowel cleansing, suggesting stability of bacterial associated urinary metabolome despite alterations in the intestinal faecal microbiome.

Both untargeted multivariate and targeted univariate analysis was performed on the faecal metabolome. In contrast to the recent study by Nagata et al. (277) that observed an immediate change in faecal metabolome after bowel cleansing (the same day as the purgatives were given), this study showed no changes at day 3 following colonoscopy. In this study PCA plots showed subject clustering in ten out of eleven study participants at all three time points, and targeted analysis showed no significant differences in ten selected metabolites following bowel cleansing.

Several limitations were present in this study, including a relatively small number of subjects - although this was a similar number to previous studies assessing the effects of bowel cleansing. Although all the colonoscopy examinations showed no significant pathology, there was a mixture of healthy subjects and subjects with underlying background pathology including quiescent ulcerative colitis. The week 6 sample cohort was incomplete, with two subjects not giving faecal samples, and one subject not giving a urine sample. The initial post-intervention sample time point was taken three days after bowel cleansing, and so more immediate perturbations in metabolites and the faecal microbiome may have been missed. NMR spectroscopy was used as the analytical platform, which gives a good overall qualitative and relative quantitative assessment of metabolites, but is of less sensitivity than mass spectrometry (160).

Timepoint selection likely affects the outcome of this study, with samples being taken three days after colonoscopy potentially too late to capture transient changes in the metabolome – this point is discussed further in Chapter 6.

In conclusion, bowel cleansing using PEG causes a temporary disturbance in bacterial alpha diversity measured in faeces, but no significant changes in the faecal and urine metabolic profile, suggesting that the faecal microbiome and its associated metabolome is resistant to the effects of an induced osmotic diarrhoea.

Chapter 6: General Discussion and Conclusion

This thesis contains three metabonomic IBD studies to further explore the application of ^{1}NMR spectroscopy in a real-life population, and specifically examining the effects of comorbidities, obesity and use of bowel purgatives.

6.1 Exploring the application of IBD urinary metabonomics to a real-life population, including subjects with comorbidities.

Previous studies of metabonomics in IBD have been conducted in very homogeneous patient populations, excluding significant comorbidities (156, 161, 207, 209-211). They consistently found that microbial associated metabolites in urine are discriminatory in IBD, along with TCA metabolites and several amino acids. This study had the aim of exploring the application of urinary ^{1}H NMR metabolic profiling on a real-life population and specifically assessing whether the discriminatory metabolites could still be observed in IBD patients with comorbidities. It took the approach of trying to recruit all comers from IBD clinics, with the exception of patients with stomas and those who were receiving (or had recently received) antibiotics or pre-/probiotics, with the aim of trying to reflect a real-life population. A large number of subjects were recruited into this study; 215 IBD patients and 100 controls. Urine was obtained from all subjects, and stool from 158 subjects. Comorbidities in recruited subjects included T2DM, asthma, hypertension / cardiovascular disease and other inflammatory conditions.

This study, and thesis, has focused on the application of urinary metabonomics in light of the ease of collecting and handling this biofluid, and the wealth of metabolic information it contains including endpoints of exogenous and microbial metabolism. The aim was to assess all subjects with both multivariate and targeted analysis of urine, and to correlate microbial associated metabolites with compositional changes in the faecal microbiome associated with

IBD to help consolidate the link between changes in the urinary metabolome and faecal microbiome in IBD.

Assessment of the faecal microbiome was restricted to IBD with no comorbidities and healthy controls. This was to make the groups more homogenous and remove potential confounding from other conditions that may impact on the baseline faecal microbiome associated with IBD. Faecal 16S rRNA analysis showed changes that have been observed in previous studies including a reduction in alpha diversity, a higher abundance of *Bacteroidetes*, a reduction of *Clostridia* species, and some specific changes such as a reduction in the *Ruminococcaceae* family and an enrichment of *Eggerthella*. A reduction in *Clostridia* species has been associated with a reduction in microbial associated metabolites hippurate and 4-cresol sulfate.

Supervised multivariate analysis of urine samples from all recruited subjects showed, as in previously studies, that resonances from 5-aminosalicylates were the principal components driving separation between IBD and control subjects. This was the first study to employ STOCSY-E, a statistical correlation tool, to try and remove the effect of 5-ASAs on the NMR spectrum. Unfortunately, due to significant 5-ASA spectral peak overlapping, these resonances could not be removed, and so subjects taking 5-ASAs were excluded from further analysis rather than removing this vital area of the NMR spectrum. The removal of patients on 5-ASAs reduced the study size significantly and consequently had a considerable impact on analysis as a significant proportion of patients, particularly UC patients, were receiving 5-ASAs. For a pre-diagnostic use, this may not necessarily be an issue, but could be a significant limitation in patients already diagnosed with IBD, particularly UC, where generally a very high population of patients take 5-ASAs.

Subsequent multivariate analysis observed differences in urinary metabolites between IBD and Crohn's disease cohorts relative to controls with changes in the microbial related metabolites

hippurate, 4-cresol sulfate and formate, the TCA metabolite citrate, and the amino acids alanine and betaine. These differences were present in models which included subjects with non-IBD health conditions.

Higher levels of phenylacetylglutamine (PAG), produced from bacterial phenylalanine metabolism (331), were seen in IBD cohorts compared to controls. PAG has not been previously observed in IBD, but known to have an inverse correlation with obesity (267, 270). The IBD cohorts in this study had a lower BMI than the control groups, and so this suggests that a difference in BMI between groups had led to this finding.

Targeted analysis of 10 metabolites associated with IBD in previous studies were examined in this study (161, 207, 209, 210). Reduced urinary excretion of 4-cresol sulfate and hippurate was observed across several comparisons, along with reduced alanine (161, 207, 209, 210). In this study, a higher concentration of citrate was seen in IBD cohorts containing subjects with comorbidities, but not in the IBD alone comparisons with healthy controls, suggesting another condition such as type 2 diabetes mellitus (previously observed to affect urinary citrate concentration (225)) may be contributing to this finding. Directly assessing IBD and control cohorts with and without the presence of non-IBD health conditions showed no changes in these targeted metabolites.

Overall, this study shows specific changes in IBD microbial and non-microbial related metabolites appear to be consistent in presence of subjects with comorbidities. However, the presence of 5-aminosalicylates is a significant confounder, and in this study STOCSY-E was not able to correct for this. This meant that to include patients taking 5-ASAs the whole aromatic region of the NMR spectrum would have to be excluded, which is a region containing many of the resonances related to intestinal microbial activity – patients taking 5-ASAs were

instead excluded from further analysis. Therefore, to be useful, ^{1}H NMR spectroscopy cannot be used on patients taking 5-ASAs.

This study also showed that obesity and type 2 diabetes mellitus may influence the metabolome of IBD subjects by altering concentrations PAG, and citrate, so future studies this would need to account for this.

There were several limitations to this study. Firstly, by trying to recruit all IBD patients (excluding those with stomas and those on antibiotics or pre-/probiotics) there will have been significant heterogeneity within comparison groups. Despite several advantages of ^{1}H NMR spectroscopy (previously described in Chapters 1 and 3), this technique is less sensitive than mass spectrometry, and with the urinary metabolome influenced by exogenous metabolites from diet and medication, this could potentially impact on results. This can potentially be overcome by analysing a large number samples to account for some of these potential confounding factors. This study initially recruited 315 patients, but could not overcome the impact of 5-ASAs on the NMR spectrum, which led to the removal of a large number of patients, as noted above.

This study only examined the faecal microbiome of non-comorbid IBD patients and healthy controls, and although this gives an insight into the potential causes of metabolomic changes being seen in the urine, it would have been more informative if all subjects were included in this part of the study. Using statistical correlation to examine the changes in faecal bacterial composition with differences expressed in the urinary metabolome associated with IBD would help to increase the confidence in the association between these two areas that were examined in this chapter.

Separately examining the effects of individual comorbidities on IBD, particularly type 2 diabetes mellitus, hypertension / cardiovascular disease, and inflammatory conditions including

asthma would be better than grouping them all together as was done in this study, but would need substantially larger numbers, and once subjects with 5-ASAs were removed, the number of participants with individual comorbidities was too low for individual assessment.

6.2 Effects of obesity in IBD patients

This chapter assessed the influence of obesity on the urinary metabolome of patients with IBD (245). Obesity rates in IBD have been growing (243), and there is increasing evidence that obesity alters the nature of this disease due to the pro-inflammatory function of adipose tissue (20, 246). The urinary metabolome has been shown to be influenced by obesity, and this includes changes in several IBD associated metabolites.

This study analysed urine samples from 42 obese IBD subjects (BMI >30 kg/m2), 35 obese controls, 95 normal weight IBD patients and 38 normal weight controls. As there is a significant overlap with obesity and type 2 diabetes mellitus, a condition also known to affect the urinary metabolome, the number of subjects with this condition was also reported and considered in the final analysis.

Unsupervised multivariate analysis observed no clustering of obese subjects, possibly due to the heterogeneity of the subjects and potentially the number of participants in the study, but could represent the fact that obesity was relatively less important in clustering of subjects compared to other factors such as IBD. Targeted univariate analysis focused on the same profile of IBD associated metabolites that were examined in chapter 3 of this thesis, but with the addition of dimethylamine, a microbial associated metabolite that has been consistently reported to be increased in obesity (267, 270). Higher concentrations of dimethylamine were associated with obesity in this study, with higher excretion in IBD obese subjects compared to normal weight IBD patients, and also in non-IBD obese subjects compared to normal weight controls. Subgroup analysis only observed different levels of dimethylamine in obese UC

patients. Reduced succinate excretion was seen in UC obese patients compared to those of a normal weight, a finding that has been previously observed in obesity (267). No changes were demonstrated in the Crohn's disease cohort when comparing obese patients relative to those with a normal weight.

Examining obese IBD patients with obese controls demonstrated a reduction in 4-cresol in UC, and differences in citrate and alanine in Crohn's disease. No change in hippurate was observed, one of the most consistently observed findings associated with IBD. Hippurate has a strong inverse correlation with BMI (267). Reduced excretion of 4-cresol sulfate and hippurate was observed in the normal weight comparison of Crohn's disease compared to controls.

Overall, comparing obese with normal weight IBD and non-IBD subjects only showed a difference in dimethylamine, which is not normally associated with IBD but has been observe to be influenced by BMI. Comparing obese IBD cohorts with obese controls showed some changes associated with IBD, but not a reduction in hippurate, which is the most consistent finding seen in IBD urinary metabolomic studies. Increased concentration in hippurate has been reported with increasing glycosuria (225), and so the increased presence of type 2 diabetes mellitus in the obese IBD and control cohorts, and the relatively lower numbers (particularly of Crohn's patients) may also be a factor in why lower hippurate excretion was not seen in the obese IBD subjects. Hippurate excretion has been observed to have an inverse relationship with BMI (267), and this may also have been lost in these data due to the above factors.

The limitations of this study include the number of study participants and heterogeneity within the comparison groups. The relatively lower number of participants, compared for instance with Chapter 3, likely restricted the use of supervised multivariate analysis in already quite heterogeneous comparison groups, as well as affecting the targeted univariate analysis. Power

calculations cannot be applied to metabonomics studies, and so the size of previous studies which have acquired positive findings is usually used as a guide to the required number of study participants – although this study was of similar size to previous studies, past investigations have had more homogeneity between groups.

Changes in the urinary metabolome have been observed in type 2 diabetes mellitus, which in turn is associated with obesity. Previously reported differences include increased hippurate, citrate, and alanine concentrations with increasing glycosuria and glycohaemoglobin (225). The presence of type 2 diabetes mellitus within obese cohorts may well have influenced the targeted analysis.

6.3 Effects of bowel cleansing on urinary metabolic profiling

In this chapter the effects of bowel purgatives on the urinary and faecal metabolome were examined, as IBD patients often undergo an induced osmotic diarrhoea with bowel purgatives before a colonoscopy. Several previous studies have examined the effect of bowel cleansing on the faecal and mucosal microbiome, and although there has been variation in the reported effects, the overall consistent findings have been a temporary perturbation of the microbiome with transient recovery (276-278, 280). Only one study (277) has examined the effects on the associated metabolome, and this was in faecal samples only, with the overall conclusion that bowel cleansing caused a change in the faecal metabolome immediately after bowel cleansing, but this had recovered at day 14 sampling.

In this study 2 litres of polyethylene glycol (PEG) was given to induce an osmotic diarrhoea in the 11 participants that were recruited, with baseline urine and faecal samples taken 3 days before colonoscopy (baseline sample), 3 days afterwards, and then 6 weeks after their procedure. Faecal samples were analysed using 16S rRNA sequencing to assess changes in

bacterial composition, and both stool and urine were analysed with ^{1}H NMR spectroscopy to investigate the impact on the corresponding metabolomes.

Faecal 16S rRNA sequencing analysis showed a decrease in alpha diversity (number of taxa and relative abundance of those taxa present) following bowel preparation, but no significant difference in beta diversity (variability of community composition). Taxonomic composition analysis showed no statistically significant changes from phylum to genus level following bowel cleansing.

Unsupervised multivariate analysis of urine samples (PCA plot) showed subject clustering of urine samples at all 3 time points in samples from 9 of the 11 study participants, and similarly there was subject clustering of faecal samples from 9 out of 10 participants. These findings were in contrast to the previous study (277) assessing the impact on the faecal microbiome, which showed subject clustering was lost immediately after bowel cleansing (day 1), but then recovered at day 14 sampling (the next time point that was assessed).

Overall, this study showed that an induced osmotic diarrhoea caused a temporary disturbance in microbiome (reduced bacterial alpha diversity), but no significant changes in the faecal and urine metabolic profile, suggesting resistance in the faecal microbiome and its associated metabolome. Taking into account the findings of the previous study assessing the impact on the faecal metabolome, this would suggest that any immediate disturbance in the metabolome recovers within 3 days of bowel cleansing (277).

Limitations of this study included the relatively small number of participants - although this was a similar number to previous studies assessing the effects of bowel cleansing. The incomplete number of samples acquired at the third time points (mainly faecal samples at the third time point) led to an incomplete dataset. There was also a mixture of healthy subjects and subjects with underlying quiescent ulcerative colitis which in itself could lead to some

confounding in the results. The initial post-intervention sample time point was taken three days after bowel cleansing, and so more immediate perturbations in metabolites and the faecal microbiome may have been missed.

Another potential confounder is the timing of sample taking. There has been great variation across previous studies, with the most recent published study by Nagata et al. (277) sampled one day before bowel cleansing, immediately after finishing bowel preparation (first motion passed after finishing consumption of PEG), and then 14 days post bowel preparation. A study by Gorkiewicz et al. (276) collected samples at day 3 post commencing bowel lavage and observed significant reduction in bacterial richness at this time point. Overall, studies that took faecal samples within 72 hours of bowel lavage recorded changes in the faecal microbiome (276-278).

A study by O'Brien et al. (282) collected samples one month before colonoscopy, one week before, and then one week, one month, and three to six months after colonoscopy – this study showed no significant impact on the microbiome.

The specific time points selected in this study were for the following reasons. 3 days before sampling provided the most recent as possible baseline sample without being affected by a low residue diet used in bowel preparation pre-colonoscopy, with dietary changes known to affect the metabolome (357). 3 days afterwards was selected as by this time most patients will be back on a normal diet, and six weeks afterwards to assess for any long-term effects.

Overall, this study suggests that disturbances in the microbiome related to bowel cleansing with PEG are short lived and recovery quickly. However, to gain a more complete picture a larger study focusing on the time immediately post-procedure may be helpful.

6.4 Lessons learnt from this MD

This project has provided much learning about the pitfalls of clinical research. Firstly, study design is imperative; the initial plan was to apply ^{1}H NMR spectroscopy to a wide group of patients that better reflected a real-life population than previous studies. One of the main potential confounders that had not been examined in previous IBD metabolomic studies had been the effect of other conditions, and recent publications already existed when I started my research showing that other conditions had an effect on the urinary metabolome, including some of the microbial associated metabolites seen to differ in IBD. I attended a weekly IBD clinic and a significant proportion of the patients had comorbidities and obesity. I took the approach of trying to recruit all patients who were willing to engage in research, and initially wanted urine and stool from every patient. I discovered that the type of sample was important, as patients are very willing to give urine (which I later discovered is much easier to handle and process in the lab compared to faeces), but much less willing to provide stool samples which often requires a second trip to the hospital. With ethical approval, we devised a system of sending patient information letters, with stool and urine pots inside, so patients could bring these to their next appointment, and the subsequent engagement in providing stool samples significantly improved.

At the outset I examined previous literature on comorbidities – with the exception of known IBD associations (extra-intestinal manifestations of IBD) there were no clear specific associations with other diseases. North West London, the catchment area for recruitment in this study, has significant heterogeneity in its IBD population (358) – it serves a large South Asian population, where comorbidities include a significant number of patients with type 2 diabetes mellitus, obesity and cardiovascular disease (359). It is not possible to conduct power calculations in metabonomic studies, and so the initial plan was to recruit all comers which

would then provide the option of subgroup analysis of particular cohorts that may then be shown to be important, aiming for a study population similar to previous studies. Later in the recruitment period I noted that type 2 diabetes and asthma appeared to be frequent comorbidities, and so I attempted to match this with non-IBD diabetic and asthmatic controls by going to respective diabetes and chest clinic. However, following the removal of subjects taking 5-ASAs due to their interferences on the NMR spectra that could not be resolved with STOCSY-E, subgroup analysis of diabetic and asthmatic groups was hindered. The only contingency was then to include all comorbidities together. Further studies of this area would need to use larger populations, stratified by individual comorbidities, or a very large trial of all comers.

This project showed me that research is vulnerable to many setbacks which included delays in getting access to analytical platforms (eg NMR machine), and not being able to overcome the impact of 5-ASAs on the urinary NMR spectrum using STOCSY-E.

With all projects of this type accurate clinical phenotyping, and collection and curation of samples are critical. In this study, the creation of the biobank left comparatively less time to analyse the data, which in metabolomics is a complex task requiring the acquisition of new skills which was a timely process. This highlighted the importance of having a well-maintained biobank, so metabolomic analysis can begin at an earlier interval during research time (perhaps of a small set of pilot data) to help acquire the skills necessary analytic skills. Further studies can now be conducted using the samples collected for this project.

6.5 Direction of future work

Urinary ^{1}H NMR spectroscopy has many advantages, including that allowing for easy acquisition and handling of samples that can then undergo high throughput analysis that then gives a wealth of metabolic information including endpoints of microbial metabolism, and has

shown consistent discriminatory metabolites in IBD. It has provided a valuable research tool exploring the complex pathogenesis with findings likely to increasingly be associated with other aspects of the interactome. It may also have the potential to stratify patients into different disease phenotypes and be used as part of treatment assessment.

However, limitations identified during this project have identified several potential issues of urinary ^{1}H NMR spectroscopy going forward. The presence of 5-aminosalicylates remains an ongoing issue when including patients on this medication in studies using ^{1}H NMR spectroscopy to assess urine. This study employed STOCSY-E (302) that was not able to overcome this issue, and so it may make this technique with urine as the biofluid difficult to use in ulcerative colitis, but this would not affect its use at the time of diagnosis. Future studies may have to use mass spectrometry with its increased sensitivity, but would be disadvantaged by its more complex and longer sample preparation and analysis (160, 162).

This study showed, particularly in the Crohn’s disease cohort, that discriminatory urinary metabolic differences were seen in cohorts reflecting more of a real-life IBD population, and these changes in the urinary metabolome were consistent with those reported in previous studies – particularly changes to hippurate, 4-cresol sulfate, citrate and alanine in multivariate analysis where comorbid patients were included, and there was a statistical difference in BMI between IBD and control cohorts.

Metabonomic research is continuing to move forward with large multi-site and multinational projects recognising the potential value metabononics in identifying risk of developing IBD, as well personalising treatment. The Crohn’s and Colitis Canada (CCC) Genetic, Environmental, Microbial (GEM) Project, a multinational project initiated in 2008 which has recruited over 5000 first degree relatives (FDR) of patients with Crohn's disease, has assessed potential metabolic markers within stool to predict onset of Crohn's disease (360). As part of the CCC-

GEM project, a recent study by Lee et al. has identified two faecal metabolic markers that confer a higher risk of developing Crohn's disease, and five markers that are protective (361). In the UK, the IBD-RESPONSE project is collecting stool, blood and tissue from multiple sites around the UK, and will use metabonomic techniques with the aim of developing algorithms to predict patients who will respond or not to specific IBD biological and small molecule treatments in Crohn's disease and UC (362).

Summarising how to make further progress the following areas need to be studied:

i. Larger real-life populations need to be studied, either with much higher recruitment using an all-comers approach, or lower numbers stratified by comorbidities, and selecting comorbidities that are of higher prevalence and known to influence the intestinal microbiome and associated metabolome.
ii. Use of a more sensitive analytical technique: with liquid chromatography–mass spectrometry (LC-MS, good detection of polar compounds common in urine samples) or gas chromatography–mass spectrometry (GC-MS, less interference of drug metabolites conjugated with target molecules) (160, 162) – a combination of NMR experiments for untargeted analysis and mass spectrometry (GC- and LC-MS) for targeted analysis was effective in the mapping of the human urinary metabolome (357)
iii. Using prospective studies of patients at the time of diagnosis and so before they are commenced on 5-aminosalicylates that interfere with the vital region of the urinary NMR metabolome.
iv. Further studies should investigate the relationship between the microbiome and discriminatory metabolites, using statistical correlation tools (can present with heat maps etc), to further assess this relationship.

Publications and conference presentations arising from this thesis to date

Original research paper publication:

Powles, S. T., et al. (2022). Effects of bowel preparation on intestinal bacterial associated urine and faecal metabolites and the associated faecal microbiome. BMC gastroenterology 22(1): 1-9.

Published conference articles:

Powles, S. T., et al. (2019). PTH-112 Effect of co-morbidities in Crohn's disease associated urinary metabolic profiles, BMJ Publishing Group.

Powles, S. T., et al. (2019). PTH-113 Effect of ethnicity on the faecal water metabolic profiles in Crohn's disease, BMJ Publishing Group

Powles, S. T., et al. (2018). "Mo1945-Effect of bowel purgatives on urinary metabolic profiling associated with the faecal microbiome." Gastroenterology 154(6): S-860.

Powles, S. T. R., et al. (2017). "The use of rapid evaporative ionisation mass spectrometry (REIMS) in faecal samples to identify inflammatory bowel disease." United European Gastroenterology Journal 5(5 Supplement 1): A500-A501.

Powles, S., et al. (2017). "P274 Assessing the individual risk of acute severe colitis at diagnosis in a South Asian population." Journal of Crohn's and Colitis 11(suppl_1): S217-S217.

Powles, S., et al. (2015). PTH-069 Effect of co-morbidities on urinary metabolic profiling in the characterisation of patients with inflammatory bowel disease, BMJ Publishing Group.

Oral presentations:

Powles, S. T. R., et al. Effect of comorbidities on urinary metabolic profiling in the characterisation of patients with inflammatory bowel disease. United European Gastroenterology Week (UEGW), 2015

Poster presentations:

Powles, S. T. R., et al. Effect of bowel purgatives on urinary metabolic profiling associated with the faecal microbiome. Digestive Diseases Week (DDW), 2018.

Powles, S. T. R., et al. Effect of ethnicity on the faecal water metabolic profiles in Crohn's disease. British Society of Gastroenterology (BSG) Annual Meeting, 2019.

Powles, S. T. R., et al. The use of rapid evaporative ionisation mass spectrometry (REIMS) in faecal samples to identify inflammatory bowel disease. United European Gastroenterology Week (UEGW), 2017, and Digestive Diseases Week (DDW), 2018.

Powles, S. T. R., et al. Assessing the individual risk of acute severe colitis at diagnosis in a South Asian population. BSG Annual Meeting, 2017, and 12th Congress of European Crohn's and Colitis Organisation (ECCO), 2017.

Appendix

Supplementary Table 1

IBD with no comorbidities v healthy controls (HC)						
Comparison cohorts	*n*	Significant differences in subject characteristics	R2X	Q2	p value CV-ANOVA	Most significant metabolites driving model*
IBD : HC	*127 (75 : 52)*	Age Ethnicity BMI	0.084	0.180	0.041	hippurate ↓ alanine ↓ citrate ↑ p-cresol ↓ PAG ↑ DMG ↓ formate ↓
CD : HC	*107 (55 : 52)*	Age Ethnicity BMI	0.085	0.231	0.014	hippurate ↓ alanine ↓ citrate ↑ p-cresol ↓ PAG ↑ DMG ↓ betaine ↓ formate ↓ leucine ↓ aspartate ↓
UC : HC	*72 (20 : 52)*	nil	0.081	0.003	NS†	
CD : UC	*75 (55 : 20)*	Operations Oral immuno-modulators Biologics	0.096	-0.04	NS	

Supplementary Table 1: OPLS-DA models comparing cohorts excluding subjects on 5-aminosalicylates and those with comorbidities. Arrows denote whether the relative abundance of metabolite is higher or lower in the IBD cohort. *NS = non-significant.

Supplementary Table 2

IBD, CD and UC with comorbidities only (c) vs healthy controls (HC) and IBD with no comorbidities (IBD)				
Comparison cohorts*	*n*	R2X	Q2	p value CV-ANOVA
IBDc : HC	*27 : 52*	0.116	-0.029	NS†
CDc : HC	*19 : 52*	0.125	0.048	NS
UCc : HC	*8 : 52*	0.091	0.151	NS
CDc : UCc	*19 : 8*	0.192	0.108	NS
IBDc : IBD	*27 : 75*	0.103	-0.129	NS
Cc : HC	*48 : 52*	0.231	-0.030	NS

Supplementary Table 2: OPLS-DA models comparing IBD subjects with a comorbidity with IBD subjects without a comorbidity, and comparing these groups with healthy controls and with non-IBD subjects with a comorbidity. *IBDc = IBD subjects with a comorbidity, CDc = CD subjects with a comorbidity, UCc = UC subjects with a comorbidity, Cc = non-IBD subjects with a comorbidity, IBD = IBD subjects with no comorbidities, HC = healthy controls †NS, non-significant

Supplementary Table 3

#	Comparison	*n*	Differentiating metabolites
1	All IBD and all controls:		
	IBD : C	*102 : 100*	↓4-cresol, ↓hippurate, ↓alanine
	CD : C	*74 : 100*	↓4-cresol, ↓hippurate, ↑citrate, ↓alanine
	UC : C	*28 : 100*	↓4-cresol, ↓alanine
	CD : UC	*74 : 28*	↓4-cresol, ↓acetate
2	IBD with no-comorbidities and healthy controls:		
	IBD : HC	*75 : 52*	↓4-cresol
	CD : HC	*55 : 52*	↓4-cresol, ↓hippurate
	UC : HC	*20 : 52*	↓4-cresol
	CD : UC	*55 : 20*	-
3	All IBD and healthy controls:		
	IBD : HC	*102 : 52*	↓4-cresol, ↓hippurate, ↑citrate
	CD : HC	*74 : 52*	↓4-cresol, ↓hippurate, ↑citrate
	UC : HC	*28 : 52*	↓4-cresol
4	IBD with comorbidities and controls:		
	IBDc : HC	*27 : 52*	↓4-cresol, ↓hippurate, ↑citrate
	CDc : HC	*19 : 52*	↓4-cresol, ↓hippurate, ↑citrate
	UCc : HC	*8 : 52*	↓4-cresol
	CDc : UCc	*19 : 8*	-
	IBDc : Cc	*27 : 48*	↓4-cresol, ↓alanine, ↓acetate
	CDc : Cc	*19 : 48*	-
	UCc : Cc	*8 : 48*	↓4-cresol, ↓alanine, ↓acetate
	IBDc : IBDnc	*27 : 75*	-
	CDc : CDnc	*19 : 55*	-
	UCc : CDnc	*8 : 20*	-
	Cc : HC	*48 : 52*	-

Supplementary Table 3: Targeted univariate analysis comparing IBD subjects and control cohorts with and without the inclusion of subjects with non-IBD health conditions. Only differences that were statistically significant ($p<0.05$) after Benjamini-Hochburg correction for multiple comparisons were included in this table. Arrows denote whether relative abundance of metabolite is higher or lower in the first comparison group. †IBD = IBD subjects with or with no comorbidities, HC = healthy controls, C = non-IBD controls including healthy controls and subjects without IBD but with another health condition, IBDc = IBD subjects with a comorbidity,

CDc = CD subjects with a comorbidity, UCc = UC subjects with a comorbidity, Cc = non-IBD subjects with a non-IBD health condition, IBDnc = IBD subjects with no comorbidities, CDnc = Crohn's disease subjects with a non-IBD health condition, UCnc = UC subjects with a non-IBD health condition ††NS, non-significant

Supplementary Table 4

IBD with comorbidities included† vs mixed controls (C)††				
	p value IBD : C *102 : 100*	p value CD : C *74 : 100*	p value UC : C *28 : 100*	p value CD : UC *74 : 28*
Microbial metabolites				
4-cresol sulfate	**<0.001**	**0.002**	**<0.001**	**0.015**
formate	0.262	0.673	0.108	0.255
hippurate	**0.004**	**0.002**	0.079	0.099
methylamine	0.945	0.604	0.645	0.360
trigonelline	0.996	0.546	0.486	0.210
SCFAs				
acetate	0.146	0.898	0.012	0.029
butyrate	0.277	0.145	0.717	0.219
TCA metabolites and amino acids				
citrate	0.151	**0.016**	0.995	0.036
succinate	0.425	0.787	0.073	0.042
alanine	**0.003**	**0.005**	0.020	0.779

Supplementary Table 4: Targeted univariate analysis of IBD subjects with no comorbidities and healthy controls. Bold figures are statistically significant ($p<0.05$) after Benjamini-Hochburg correction. †group includes IBD alone and IBD with a comorbidity ††group includes healthy controls and non-IBD patients with a comorbidity. Arrows denote whether relative abundance of metabolite is higher or lower in corresponding cohort.

Supplementary Table 5:

IBD with comorbidities included† vs healthy controls (HC)			
	p value IBD : HC *102 : 52*	p value CD : HC *74 : 52*	p value UC : HC *28 : 52*
Microbial metabolites			
4-cresol sulfate	**<0.001**	**0.006**	**<0.001**
formate	0.078	0.240	0.035
hippurate	0.033	**0.012**	0.191
methylamine	0.812	0.846	0.488
trigonelline	0.282	0.134	0.699
SCFAs			
acetate	0.345	0.914	0.084
butyrate	0.794	0.578	0.901
TCA metabolites and amino acids			
citrate	**0.016**	**0.013**	0.230
succinate	0.866	0.564	0.318
alanine	0.094	0.091	0.181

Supplementary Table 5: Targeted univariate analysis of IBD subjects including those with a comorbidity and healthy controls. Bold figures are statistically significant ($p<0.05$) after Benjamini-Hochburg correction; †group includes subjects with IBD alone and those with IBD and a comorbidity. Arrows denote whether relative abundance of metabolite is higher or lower in corresponding cohort.

Supplementary Table 6:

IBD with comorbidities only (c)† vs healthy controls (HC)				
	p value IBDc : HC *27 : 52*	p value CDc : HC *19 : 52*	p value UCc : HC *8 : 52*	p value CDc : UCc *19 : 8*
Microbial metabolites				
4-cresol sulfate	**<0.001**	**0.016**	**<0.001**	0.013
formate	0.056	0.093	0.125	0.863
hippurate	0.053	0.104	0.107	0.545
methylamine	0.087	0.181	0.129	0.914
trigonelline	0.370	0.127	0.929	0.152
SCFAs				
acetate	0.043	0.396	0.015	0.137
butyrate	0.234	0.587	0.166	0.948
TCA metabolites and amino acids				
citrate	0.055	**0.002**	0.743	0.010
succinate	0.994	0.252	0.333	0.027
alanine	0.055	0.308	0.035	0.298

Supplementary Table 6: Targeted univariate analysis using a Mann-Whitney U test of IBD subjects with no comorbidities and healthy controls. Bold figures are statistically significant ($p<0.05$) after Benjamini-Hochburg correction. Arrows denote whether relative abundance of metabolite is higher or lower in corresponding cohort. †IBDc = IBD subjects with a comorbidity, CDc = CD subjects with a comorbidity, UCc = UC subjects with a comorbidity, HC = healthy controls.

Supplementary Table 7

IBD with comorbidities only (c)† vs non-IBD controls with at least one comorbidity (Cc)			
	p value IBDc : Cc *27 : 48*	p value CDc : Cc *19 : 48*	p value UCc : Cc *8 : 48*
Microbial metabolites			
4-cresol sulfate	**<0.001**	0.046	**<0.001**
formate	0.760	0.778	0.815
hippurate	0.028	0.067	0.062
methylamine	0.292	0.453	0.315
trigonelline	0.338	0.973	0.123
SCFAs			
acetate	**0.014**	0.447	**0.001**
butyrate	0.028	0.106	0.039
TCA metabolites and amino acids			
citrate	0.258	0.017	0.809
succinate	0.396	0.609	0.075
alanine	**0.002**	0.0536	**0.002**

Supplementary Table 7: Targeted univariate analysis using a Mann-Whitney U test of IBD subjects with no comorbidities and healthy controls. Bold figures are statistically significant ($p<0.05$) after Benjamini-Hochburg correction. Arrows denote whether relative abundance of metabolite is higher or lower in corresponding cohort. †IBDc = IBD subjects with a comorbidity, CDc = CD subjects with a comorbidity, UCc = UC subjects with a comorbidity, Cc = non-IBD subjects with another health condition

Supplementary Table 8:

IBD with comorbidities only (c)† vs non-comorbid IBD groups (nc), and non-IBD controls with at least one comorbidity (Cc) vs healthy controls (HC)				
	p value IBDc : IBDnc *27 : 48*	p value CDc : CDnc *19 : 48*	p value UCc : UCnc *8 : 48*	p value Cc : HC
Microbial metabolites				
4-cresol sulfate	**0.013***	0.536	**0.029***	0.609
formate	0.457	0.223	0.785	0.185
hippurate	0.805	0.780	0.273	0.749
methylamine	**0.008***	0.056	0.090	0.648
trigonelline	0.911	0.500	0.501	0.079
SCFAs				
acetate	**0.010***	0.296	**0.026***	0.776
butyrate	0.113	0.837	**0.012***	0.392
TCA metabolites and amino acids				
citrate	0.209	**0.033***	0.780	0.477
succinate	0.627	0.263	0.746	0.456
alanine	0.331	0.651	0.069	0.258

Supplementary Table 8: Targeted univariate analysis using a Mann-Whitney U test of IBD subjects with no comorbidities and healthy controls. Figures in bold have a p value less than 0.05. *Not statistically significant when corrected for multiple comparisons using the Benjamini-Hochburg correction. Arrows denote whether relative abundance of metabolite is higher or lower in corresponding cohort. †IBDc = IBD subjects with a comorbidity, CDc = CD subjects with a comorbidity, UCc = UC subjects with a comorbidity, IBDnc = IBD subjects with no comorbidities, CDnc = Crohn's disease subjects with a non-IBD health condition, UCnc = UC subjects with a non-IBD health condition, Cc = non-IBD subjects with another health condition. ††NS, non-significant

Supplementary Table 9:

Subject characteristic comparisons - univariate analysis – p values				
	IBDc : IBDnc	CDc : CDnc	UCc : UCnc	Cc : HC
n	*27 : 75*	*19 : 55*	*8 : 20*	*48 : 52*
Age (median)[1]	**<0.001**	**0.003**	**<0.001**	**0.035**
Sex[2]	0.282	0.073	0.394	0.236
Ethnicity (Cau : SA : Oth)[*2]	**<0.001**	**0.040**	**0.005**	0.196
BMI (median)[1]	**0.008**	0.198	**0.038**	0.776
Non-IBD medication[2]	**0.023**	**0.008**	**0.002**	**<0.001**

Supplementary Table 9: Characteristic comparisons between subject cohorts. [1]Mann-Whitney U test, [2]Chi-squared test, p value of <0.05 considered significant and are in bold.

Supplementary Table 10:

Lifestyle comparisons - univariate analysis – p values				
	IBD obese v normal weight	Non-IBD obese v normal weight	Obese IBD v obese controls	Normal weight IBD v normal weight controls
n	*36 : 100*	*35 : 38*	*36 : 35*	*74: 38*
Vegetarian Smoker Exercise	0.101 0.214 0.434	0.821 0.351 0.055	0.256 0.701 0.725	0.546 0.623 0.091
Consumed within the last 24 hours prior to producing samples:				
Meat	0.339	0.225	0.943	0.502
Fish	0.874	0.443	0.773	0.082
Cheese	0.465	0.255	0.801	0.931
Grapefruit	0.131	0.738	0.679	0.388
Cherries	0.718	0.436	0.477	0.381
Liquorice	0.640	0.370	0.533	0.187
Walnuts	0.903	0.124	0.635	0.179
Vanilla	0.707	0.530	0.042	0.395
Milk	0.262	**0.028**	0.079	0.772
Yoghurt	0.097	0.217	0.840	0.632
Berries	0.035	0.909	0.309	0.343
Carbonated drinks	0.772	0.952	0.741	0.273
Coffee	0.491	0.557	0.492	**0.022**
Tea	0.399	0.416	0.100	0.213
Herbal tea	0.594	0.378	0.219	0.470
ETOH	0.213	0.570	0.180	0.509

Supplementary Table 10 – Statistical analysis of lifestyle comparisons between subject cohorts. Chi square test, p value of <0.05 considered significant and are in bold.

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