Optimising the medical management of ileoanal pouch related complications and discovering novel therapeutic avenues through metabonomic profiling

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For Cynthia and Esha.

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Declaration of originality and contributions to this thesis

This thesis contains work that has been performed by me under the supervision of Professor Ailsa Hart, Professor Susan Clark, Professor Elaine Holmes and Professor Julian Marchesi.

All processing of data, statistical analysis and data interpretation was undertaken by me. A medical statistician, Paul Bassett, checked that the statistical analysis was correct. Magali Sarafian, Alexandros Pechanvalis and Ivan Contreras supported me in my analysis of NMR and mass spectrometry. Further supervision was provided by Professor Elaine Holmes. Benjamin Mullish and Professor Julian Marchesi taught me how to analyse my 16s data which were processed and analysed by me.

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Declaration of copyright

Publications and presentations

Publications from thesis

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Abstract

Restorative proctocolectomy is considered a quality of life surgical procedure in patients with ulcerative colitis who fail to respond to conventional medical therapies and in some patients with Familial Adenomatous Polyposis.

This thesis explores the current management of chronic primary idiopathic pouchitis through a systematic review and meta-analysis. Following this review I have explored the clinical utility of antibiotics and biologics in a cohort of patients with both chronic primary idiopathic pouchitis and pre-pouch ileitis. I have found that the treatment options for chronic pouchitis and pre-pouch ileitis are limited and that long-term treatments such as antibiotics and biologics are ineffective in a significant proportion of patients often leading to a permanent ileostomy.

I have also explored the effect of some non-medical therapies including biofeedback and the Renew® anal insert for incontinence and evacuatory problems and have shown that they may be a useful adjunct in the treatment of these pouch related complications.

The second focus of the thesis is to try and understand the mechanisms that drive the development of pouchitis. I undertook a systematic review to explore what was already known about the gut microbiota and its role in health and disease of the pouch. I then utilised next generation sequencing technologies to include metataxonomics, nuclear magnetic resonance and mass-spectrometry gas chromatography to link the gut microbiota with the metabolic signatures in serum, urine, faeces and mucosal tissue. I used these techniques to compare patients with pouchitis against healthy controls and patients with Familial Adenomatous Polyposis.

These studies have highlighted the importance of the Firmicutes phylum and their role in the production of short chain fatty acids. I have found that a depletion in short chain fatty acids may contribute to the development of pouchitis. Future work may build on methods to increase short chain fatty acid delivery to the pouch through methods such as dietary interventions, distal feeding prior to continuity surgery or direct short chain fatty acid supplementation delivered topically to the pouch.

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List of abbreviations

AM	anorectal manometry
AP	ano physiology studies
ASCA	anti-*Saccharomyces cerevisiae* antibody
ATZ	anal transition zone
CD	Crohn's disease
CLC	Charcot-Leyden crystal protein
COG5	component of oligomeric golgi complex 5
CRP	c reactive protein
CV-ANOVA	coefficient of variation analysis of variance
EAUS	endoanal ultrasound scan
ES	external sphincter
ESBL	extended Spectrum Beta-Lactamase
ESI	electron spray ionization
EUA	examination under anesthetic
FAP	Familial adenomatous polyposis
FID	free induction delay
GC-MS	gas Chromatography-Mass Spectrometry
GPC	glycerophosphocholine
GTAC	gene therapy advisory committee
IBD	inflammatory bowel disease
IC	indeterminate colitis
ICIQB	Incontinence disorder using objective scoring system
IFX	infliximab
IBDU	inflammatory bowel disease unclassified
RPC	ileal pouch anal anastomosis

IPS	irritable pouch syndrome
IRA	ileorectal anastomosis
IAS	internal anal sphincter
IS	internal standard
ITPRIPL2	inositol 1,4,5-trisphosphate receptor interacting protein-like 2
JRES	J-resolved spectroscopy
LC-MS	liquid Chromatography-Mass Spectrometry
MCT	monocarboxylate transporters
MeSH	medical Subject Heading
mPDAI	modified PIP Disease Activity Index
MS	mass spectroscopy
MTBE	methyl *tert*-butyl ether
NICE	National Institute for Health and Care Excellence
NMDS	non-metric multidimensional scaling
NMR	nuclear magnetic resonance
NSAID	nonsteroidal anti-inflammatory drug
OPLS	orthogonal partial least squares discriminant analysis
OPLS-DA	orthogonal partial least squares discriminant analysis
pANCA	perinuclear antineutrophil cytoplasmic antibodies
PC	principle component
PCA	principle component analysis
PDAI	pouch disease activity index
PIP	primary odiopathic pouchitis
PLS	partial least squares
PLS-DA	partial least squares- discrimination analysis
PPI	pre-pouch ileitis

PPM	parts per million
QC	quality control
QOL	quality of life
RCF	relative centrifugal force
Robins-I	The Risk of Bias In Non-randomized Studies of Interventions
RPC	restorative proctocolectomy
SCFA	short chain fatty acids
SCMT	sodium-coupled monocarboxylate transporters
SMCT1	sodium coupled monocarboxylate transporter 1
SOQCS	Study of Quality of Life and Healthcare Utilisation in Patients with Ulcerative Colitis Undergoing Ileal Pouch Surgery: Prospective Observational Study
SPP.	Several species
STAMP	statistical Analysis of Metagenomic
STOCSY	Statistical Correlation spectroscopy for metabolite
STORM	subseT Optimization by Reference Matching
TBDMSCl	N-tert-Butyldimethylsilyl-N methyltrifluoroacetamide
TEMS	transanal endoscopic microsurgery
TI	terminal ileum
TSP	3-trimethylsilyl-[2,2,3,3,-$^{2}H_{4}$]-propionic acid sodium salt
TSP	tetradeuteropropionic acid
UC	ulcerative colitis
UCEIS	ulcerative colitis endoscopic index of severity
VIP	variable importance on projection

Chapter 1

Introduction: The pouch behaving badly

1.1 History of restorative proctocolectomy

1.1.1 Ulcerative Colitis

Ulcerative colitis (UC) is the most common type of inflammatory disease of the bowel, with an incidence of 10 per 100,000 people annually, and a prevalence of 243 per 100,000. This amounts to approximately 146,000 patients in the UK with a diagnosis of ulcerative colitis[1]. The disease falls under the umbrella term inflammatory bowel disease that covers both UC and Crohn's disease. UC follows a bimodal age distribution with diagnosis of the disease being most common between 15- 25 years old and 55-65[2]. Ulcerative colitis is characterised by diffuse mucosal inflammation of the colon and rectum . The disease can be broadly split up into distal disease which is usually confined to the rectum and sigmoid colon, left sided colitis which affects the left colon or pancolitis which affects the whole colon[3]. Its seminal symptoms include, diarrhoea that is often bloody, abdominal pain, urgency as well as systemic effects such as anaemia, lethargy and malabsorption. The diagnosis of UC is based on history, examination and investigations. A salient investigation is via endoscopy where criteria such as the Mayo score[4] or UCEIS[5] (ulcerative colitis endoscopic index of severity) are used to assess severity. The microscopic diagnosis of UC is based on the widespread crypt architectural distortion, a diffuse transmucosal inflammatory infiltrate with basal plasmacytosis which can eventually lead to cryptitis and crypt abscesses[6].

Acute severe colitis is defined by the presence of more than six bloody stools per day along with any one of the following: tachycardia > 90 bpm, fever > 37.8 °C, Haemoglobin < 10.5 gm/dL, and/or ESR > 30 mm/h (Truelove and Witts criteria) and is considered a medical emergency[7]. Prior to the seminal paper on steroid use in ulcerative colitis it carried a mortality of around 22%-75% within the first year[8]. After three days of medical therapy with steroids the Travis criteria suggested that 85% of

patients with more than eight stools per day, or a stool frequency between three and eight together with a CRP > 45 mg/l, would require a colectomy[9].

For those patients with UC in remission, medical options to help maintain remission include the use of both topical and systemic 5-aminosalicylate acids, immunomodulators such as azathioprine and mercaptopurine and biologics including Infliximab and Adalimumab[10]. There are many other medications in the trial setting currently being explored.

Despite medical therapy, proctocolectomy is necessary in 10-30% of patients after a decade of disease[1]. There has been an evolution in the operations available to patients with ulcerative colitis which have over time reduced in morbidity and mortality.

In 1944 Strauss established the proctocolectomy with an end ileostomy for severe colitis but was associated with a high morbidity and mortality related to the ileostomy[11]. Brooke in 1952 improved this technique by creating an everted ileostomy which significantly lowered mortality[12]. The problem with these ileostomies is that they were incontinent. To solve this Kock in the late 1960s created a continent ileostomy with an intra-abdominal reservoir[13]. This was further modified with a nipple valve which further aided continence[14].

The above operations significantly reduced mortality in those with severe UC, however patients were left with an ileostomy. The next challenge was to restore the bowel to continuity termed restorative proctocolectomy. The first attempt at this was attempted in 1948 by Devine called the ileorectal anastomosis (IRA)[15], it was Aylett in the 1960s that championed this for UC patients[16]. This procedure essentially anastomosed the small bowel to the rectum. This fell out of fashion for ulcerative colitis due to the inflammation of the rectal stump leading to excision and cancers. The next part of the thesis describes the path that led to the development of the restorative proctocolectomy with an ileo-anal pouch.

1.1.2 Familial Adenomatous Polyposis

Familial adenomatous polyposis (FAP) is an autosomal dominant disease caused by a mutation in the APC gene[17][18]. In the UK, Reed and Neel presented a detailed genetic study in 1955 and calculated the incidence of FAP at birth to be 1:8,300[19].

The disease is clinically defined by hundreds of adenomas in the colon and rectum which over time inevitably lead to formation of colorectal cancer. High-grade dysplasia or carcinoma can occur at a very young age but this is usually less common with the average age of colorectal cancer development around the age of 40 years if left untreated[20]. Therefore, prophylactic surgical treatment to prevent the development of colorectal cancer is required.

Nissen published the first surgical procedure for what is now known as FAP in 1933. They performed a straight ileoanal anastomosis in a 10 year old patient[21]. It was Lloyd- Davis that performed the first colectomy and ileorectal anastomosis for FAP at St Mark's Hospital in 1948[22]. This was the main prophylactic treatment until the development of the Ileoanal pouch described by Parks and Nicholls in 1978[23]. The next part of the thesis describes restorative proctocolectomy with the ileoanal pouch anastomosis.

1.2 Restorative proctocolectomy with Ileoanal pouch anal anastomosis

It is over 40 years since Parks and Nicholls first reported restorative proctocolectomy with ileoanal anastomosis (RPC) in the British Medical Journal[23]. Restorative proctocolectomy with ileal pouch anal anastomosis (RPC) is considered the procedure of choice in patients with refractory UC and in some patients with FAP. It is an operation that removes the large bowel and rectum and uses the patient's own small bowel as a reservoir for faeces. This approach is popular since it restores intestinal continuity and avoids a long-term stoma. Although most patients benefit from good long-term intestinal function and quality of life, complications occur in 21%-52%[24–28] of cases.

The original pouch described in 1978 was constructed using a handsewn method. The first pouch was configured as an "s" shape and was accompanied by a mucosectomy.

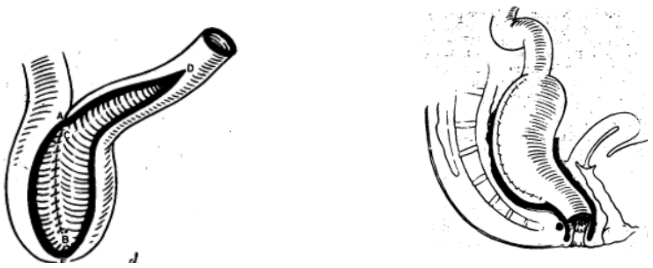

Figure 1. The original pouch

Reproduced with permission from[23].

There have been many modifications to the original pouch. The most noticeable of these is the shape of the pouch to include 'J' shaped pouch[29] and "W' shaped pouch[30]. In 1989 Kmiot developed a 'J' pouch with a bowel stapling device and demonstrated that this could be achieved without prior mucosectomy[31]. This meant that the procedure was technically less demanding and therefore the 'J' pouch became the most common. There have been various publications outlining the pros and cons of each design. One study suggested that whilst the 'J' pouch was the most common, the 'K' was associated with better function[32]. A randomised control trial that compared 'J' vs 'W' pouch design suggested that the 'J' pouch was probably the better design based on ease of performing the surgery[33]. There are limited long-term data comparing pouch designs where one study suggested that the S pouch were 8 times more likely to have pouch-related mechanical/structural complications than those with J pouches but were less likely to suffer with primary idiopathic pouchitis (PIP)[34]. In 2007 a meta-analysis comparing three pouch designs (J,W,S) concluded that all had similar perioperative complication rates. Specifically, the S pouch was associated with the need for anal intubation and the J and W pouch were associated with less frequency and less need for antidiarrhoeal agents[35]. In summarising the literature, the J pouch is probably the most practical with various pros and cons for each pouch design.

1.3 Techniques for Restorative proctocolectomy

There is a variety of surgical techniques that can be used to create the ileoanal pouch including open, laparoscopic, single incision, laparoscopic as well as various methods of anastomosis. However, further discussion of these is beyond the scope of this thesis.

1.4 Restorative proctocolectomy: agreed indications

1.4.1 Ulcerative Colitis

Patients with UC may undergo colectomy for medical refractory disease, dysplastic changes or acute severe colitis. For those wishing to avoid a permanent ileostomy after colectomy a patient may choose a 'restorative' option, including ileal pouch, IRA or continent ileostomy/Kock pouch. An ileal pouch is the most popular restorative option internationally and has been described after colectomy for all the scenarios mentioned above.

1.4.2 Familial adenomatous polyposis

Due to the high incidence of colorectal cancer in patients suffering from familial adenomatous polyposis (FAP) prophylactic colectomy is recommended. The three restorative options available are the same as those for UC. Total colectomy with IRA is used more frequently when the rectal polyp burden is low as this avoids the need for pelvic dissection, particularly in a young cohort. Despite regular surveillance 15-20 years ago the risk of rectal cancer in patients having an IRA was 13%-25% after 15-30 years[36–38] with genetic testing and good endoscopic follow up the cumulative risk is as low as 9%[36]. Restorative proctocolectomy is the restorative treatment of choice in patients perceived to be at higher the risk of carcinoma in the rectum. This includes, those with genetically increased risk, and those with a higher density and size of adenomas in the rectum[39,40].

1.5 Relative contraindications

1.5.1 Inflammatory bowel disease unclassified (IBDU)

In 10-15% of cases of acute colitis the histopathological diagnosis is equivocal between UC and Crohn's disease (CD)[41]. Historically this was labelled indeterminate colitis (IC)[41] and refers to the presence of pathological features of both UC and CD in the same colectomy specimen affected by severe inflammation. The accepted terminology is now called IBDU[10]. Once the acute phase has resolved either by medical treatment or emergency colectomy, RPC may be justified in this circumstance provided that there is no clinical suspicion of CD, whether such as perianal disease or evidence of proximal gastrointestinal inflammation[42]. RPC in IBDU results in morbidity and failure rates similar to patients with UC[43]. Full

preoperative counselling regarding the possibility that the diagnosis may be CD is essential.

Patients with IBDU comprise 10–15% of all patients undergoing RPC[44] and in 15-20% of these patients an initial diagnosis IBDU may change to CD after RPC[45]. In one study of patients who underwent RPC with IBDU there was a complication rate of 50% versus 3% in a comparison UC group ($p<0.001$)[46]. A further study suggested patients with IBDU had significantly more episodes of pelvic sepsis (17% IBDU *versus* 7% UC), pouch fistula (31 *versus* 9%), and pouch failure (27 *versus* 11%)[47]. Tekkis *et al* [48] compared patients with IBDU favouring CD *versus* IBDU favouring UC. Patients with IBDU or IBDU favouring UC (group 1, n = 26) had a pouch failure rate of 11.5% versus 57.5% for patients with CD or IBDU favouring CD [48]. Despite these results they concluded that the functional outcome was still similar in the two groups and that patients with IBDU should still remain candidates for RPC with careful pre-operative counselling[48].

1.5.2 Crohn's Disease

Restorative proctocolectomy has been considered to be inappropriate in patients with an original diagnosis of CD[49]. Reasons for this include an increased rate of pouch dysfunction, fistula formation, strictures of the pouch, abscess formation, peri-pouch sepsis and development of short bowel[47,50,51]. Crohn's disease of the pouch is a poorly defined entity, and can be difficult to diagnose and challenging to treat[52].

Despite a high incidence of complications, several studies have demonstrated benefits of RPC for CD. Panis *et al*[53] highlighted that in the absence of perianal or small bowel disease, RPC could be performed in patients with similar outcomes found in those who have a pouch for UC [53]. Regimbeau *et al*[54] reported that in the absence of small bowel disease, RPC can be safely performed with limited morbidity at ten year follow-up[54]. Phillips[49] suggests that it is unjustified to compare complication rates in RPC for CD with RPC for UC or FAP as they are completely different conditions. Phillips[49] highlighted that any surgery for CD has more complications due to the nature of the disease and that RPC should not immediately be discounted in patients without small bowel or anal disease[49].

In view of this, the general approach to known CD is to avoid RPC. In those who originally undergo RPC for presumed UC, the incidence of a change to a diagnosis of CD is 2-8%[47]. In patients with IBDU RPC is generally not considered due to concern that the disease will eventually develop into CD. Indeed, studies report that an initial diagnosis IC has a 15-20% of being changed to a diagnosis of CD after RPC[45].

Despite diagnostic advances, CD of the pouch can be difficult to detect and predict. In patients with RPC, *de novo* CD can develop weeks to years later, even when histopathological reassessment of the proctocolectomy clearly shows UC[55].

1.5.2.1 Defining CD of the pouch

Patients with CD of the pouch can have a varied presentation. Symptoms may include abdominal pain, urgency, increased stool frequency, incontinence, seepage and extra-intestinal manifestations such as joint pains and rashes.

Criteria that have been used to diagnose CD of the pouch include: inflammation of the pouch that is resistant to antibiotic treatment, stricturing of the afferent limb, stricturing of the small bowel or fistulating disease[56–59].

Endoscopic assessment can help in the diagnosis of CD on pouchoscopy. Features include discrete small and large mucosal ulcers, loss of vascular pattern, spontaneous bleeding and friability, exudates and inflammatory pseudopolyps in the pouch, cuff, or neo-terminal ileum[55]. The presence of pre-pouch ileitis (PPI) is controversial with some studies suggesting this may be an endoscopic feature of CD[60,61]. PPI has no standard definition but is considered when there is inflammation that is proximal to the pouch. It has been reported that PPI occurs in patients with CD and not UC[60,61]. In these studies, CD was defined as ulcerated lesions of the small bowel or afferent limb[60], or the presence of non-necrotising granulomas or transmural lymphoid aggregates in the colectomy specimen[61]. Other reports have suggested that PPI is not associated with CD[62].

Histological findings that help distinguish CD have been described. Weber *et al*[63] found that pyloric gland metaplasia which was first described by Liber *et al*[64] is a potential histological marker that can distinguish between chronic UC PIP and CD. Argarwal *et al*[65] supported this finding and in addition suggested that high titres of

anti-*Saccharomyces cerevisiae* antibody (ASCA) were associated with CD[65]. The presence of granulomas still represents the most accurate histological finding to help diagnose CD. Shen *et al* [59] found that in only 10%-20% of histological samples granulomas were found[59].

In conjunction with endoscopic and histological features, radiology can be a useful adjunct to help diagnose CD. The most common modalities used are MRI and CT scanning. These can help pick up small bowel strictures, the presence of fistulae and perianal disease which all may suggest a diagnosis of CD.

1.6 Factors associated with a badly functioning pouch

1.6.1 Primary sclerosing cholangitis

Patients with primary sclerosing cholangitis who undergo restorative proctocolectomy have an increased risk of postoperative sepsis, higher long-term mortality and PIP[66,67], but a similar quality of life and function to those undergoing RPC without PSC[66]. Penna et al suggested that PSC was strongly correlated with the development of PIP and that there is a common link in their pathogenesis. However, in the largest published series of patients undergoing RPC prior to liver transplantation for PSC, the outcome was comparable to patients who had PSC without previous RPC[68].

1.6.2 Fistulating disease

Fistulating disease of the pouch carries significant morbidity and is a major cause of pouch failure[69]. The presence of fistulating disease does not necessarily confirm a diagnosis of CD as surgical complications such as wound dehiscence, anastomotic leaks and iatrogenic bowel injury can contribute to fistulating disease.

Pouch fistulae may occur at any time following restorative proctocolectomy, with an incidence of 2.6%–14%, depending on the length of follow-up[70–73]. Fistulae have been associated with a high chance of pouch failure, with studies suggesting a pouch failure rate of 21-30% following fistula formation in the pouch[72–74]. Common locations of fistulae include pouch-vaginal fistulae, perianal fistulae, pouch-cutaneous fistulae and pouch-bladder fistulae[75].

The timing of fistula formation can help aid in the diagnosis, with early fistula formation in a patient with presumed UC, more likely to represent a complication following surgery with later fistula formation in the absence of sepsis and leaks more likely to represent an inflammatory process such as CD[48,76].

Anatomical location can also help determine the aetiology of the pouch fistula. Pouch fistulae associated with the anastomosis are more likely to represent surgical aetiology whereas more complex fistulae including those found in the anal canal are likely to be more associated with CD[55].

Furthermore, response to medical treatments including antibiotics and biologics has been suggested to be an important factor in aiding diagnosis[52]. Fistulae that respond to these medical therapies are likely to be inflammatory in nature[52].

1.6.3 Pouch-vaginal fistulae

The overall risk of pouch-vaginal fistula after RPC varies between 4% and 16%, with pouch failure occurring in 21% to 30% of these patients[77]. The natural history of pouch vaginal fistula has been poorly studied; however it has been reported that these are strongly associated with CD of the pouch[78–80]. Heriot *et al*[81] found that the majority of pouch vaginal fistulae (76 %) originated from the pouch-anal anastomosis. Surgical risk factors include injury to the vagina or rectovaginal septum during pelvic dissection[82], J-pouch design[83], hand sewn anastomosis[76], entrapment from the circular stapling device,[84] anastomotic dehiscence and pelvic sepsis[79].

1.7 Contraindications to restorative proctocolectomy

1.7.1 Carcinoma in the low rectum

If a pouch is to be undertaken for patients with confirmed low rectal cancer then the rectum must be excised as per total mesorectal excision principles. Forming a pouch for a very low rectal cancer within two centimetres of the dentate line is a contraindication [85–87].

The effect the potential modifications of an oncologically sound procedure may have on pouch function are not fully understood[88]. There are small series that report the outcome of RPC for rectal cancer. Taylor et al[86] reported 17 patients with UC or FAP

complicated by adenocarcinoma (six of whom had rectal cancer) who underwent RPC. They demonstrated acceptable functional results, but advanced rectal cancer requiring adjuvant radiation had a negative impact on bowel function. A study by Radice *et al*[85] demonstrated that pouch failure was commoner in the presence of colorectal cancer compared to RPC patients without cancer (16% vs 7%, $p < 0.01$), but there was no significant difference in the oncologic outcome or long-term function. They attributed the observed increase in pouch failure to radiotherapy and progression of disease. A further study by Marchea et al[89] included 11 patients who underwent RPC for rectal cancer and demonstrated similar overall failure rates and higher with more locally advanced tumours. They suggested furthermore that restorative proctocolectomy can be successful in highly selected patients with early stage tumours.

1.7.2 Anal sphincter function

Patients with inadequate sphincter function have a higher chance of failure after RPC, particularly where the anal resting pressure is less than 40mmHg and the squeeze pressure is less than 100mmHg [72]. Pre-operative manometry does not, however, always correlate with the function after ileostomy reversal [90]. Patients at higher risk of inadequate sphincter function such as the elderly and women with previous obstetric trauma should be carefully assessed with a detailed work-up and pre-operative counselling. It is advised that elective caesarean section should be advised in patients having had RPC to diminish the chance of pelvic floor damage.

1.7.3 Acute severe colitis

Restorative proctocolectomy is contraindicated in acute severe UC as it has been demonstrated that there is a decrease in morbidity and mortality by avoiding rectal excision in this setting[91,92]. Furthermore, subtotal colectomy with end ileostomy is the procedure of choice for acute severe colitis[91,92].

1.8 Reported outcomes following restorative proctocolectomy

1.8.1 Function

Most patients can expect good long-term bowel function. In the first reported long-term follow-up in patients undergoing RPC it was found that after a mean follow-up of

99.3 months, 83% of patients had spontaneous evacuation, with 47% of patients having no night bowel motions and only 8% having urgency[93]. More recent studies have shown similar results with a median 24 hour stool frequency of four to eight, with roughly half of patients needing to defecate at night [35,70,94]. In the largest series of ten units across the UK it was found that with a median follow-up of 54 months, the median frequency was five including one motion at night, with urgency experienced by 5.1% of patients at 1 year rising to 9.1% at 15 years (P = 0.022).

Rates of urgency are 5% and this increases to 19% at 16 years[95]. Brandsborg *et al*[96] reported significantly more urgency in females when compared to males (56% vs 44% p=0.0021) [96]. Anal seepage occurs in 4% during the day and 8% at night at 5 years and this increases to 20% and 15% respectively at 20 years[35]. Pad use occurs in 2.5% during the day and 5 % at night and these rates increase to 13% and 18% at 20 years[35].

In a large cross-sectional Danish study of 1047 patients with a median follow up 11 (range, 1-30) years, there was a significant gender difference in the median frequency of defaecation in a 24-hour period. The median stool frequency for females was reported as seven [1–23] and six [1–20] for male patients (P < 0.001). The authors also noted a significantly higher incidence of major incontinence (P = 0.009) and use of pads (P = 0.01) among patients operated on 21–30 years previously than 11–20 years previously[96]. Pad usage was also significantly more common in patients operated on 21–30 years previously compared with 0–10 years previously (P = 0.003)[96].

Rates of faecal seepage and leakage can be higher in patients with pre-existing sphincter damage and can occur in up to 30% of women following vaginal delivery[97]. An occult sphincter injury may become symptomatic later as sphincter function deteriorates over time[97]. For this reason, delivery by Caesarean section is recommended to the majority of pregnant women with a pouch. Erectile dysfunction occurs in 1–2%, and failure of ejaculation in 3–4% of patients following RPC[98].

1.9 Failure

Failure is defined as the interruption of anal function through the need for pouch excision or with a defunctioning ileostomy where there is little prospect of it being

closed[99]. Failure is progressive with cumulative rates of 3.5% to 5% at 5 years and 8-16% after 20 years in large series[35,72,99–102]. Approximately 25% of pouch failures occur within 12 months of ileostomy closure and are usually the result of surgical complications such as anastomotic leakage or stricture formation, pelvic sepsis, perineal sinus and fistula formation[72,99,103]. There are three main causes of failure[66]. These include sepsis, which accounted for 80% of failures[66], the rest are due to unexplained poor function [101,104,105] and PIP [93,100,106] there is also some overlap between causes of failure. Failure after 12 months following ileostomy closure often results from refractory PIP, cuffitis, pouch strictures, prolapse, CD of the pouch or pouch vaginal fistula[72,107–109].

1.9.1 Pouch failure in Crohn's disease

Despite attempts to salvage a CD pouch, it has been estimated that pouch excision rates are 45-55% in patients with pre-operative CD[45,47]. Whilst there is no absolute indication for pouch excision in CD of the pouch, a joint decision with the patient and the multi-disciplinary team to include physicians, surgeons and nurses is essential. Reasons to consider pouch failure include; symptoms which are not tolerable to the patient, medically refractory disease, signs of persistent metabolic disease, failure to thrive, malnourishment, and overall poor quality of life. In this instance, pouch excision or an ileostomy should be offered.

Pouch excision, has been associated with a 1.5% risk of death and a high rate of early and late morbidity[110]. Complications of pouch excision include small-bowel obstruction with pelvic sepsis which often require further laparotomy[110]. In a more recent study of 84 patients, 57% of them experienced short term (<30 days) postoperative complications. The most common of these were surgical site infection and a return to theatre for perineal wounds[111]. In a recent series of 92 patients who underwent pouch excision the rate of perianal would healing following excision was reported as 78%[112].

1.10 Mortality and morbidity

1.10.1 Mortality

The mortality rate after RPC is less than 1%[113–116]. This low mortality rate is likely due to the majority of RPC procedures being carefully planned elective operations, and in some cases performed in a relatively young patient population.

1.10.2 Post-operative anastomotic leakage and pelvic sepsis

The overall incidence of pouch-related septic complications is 15-20%[116–119]. Sepsis can be acute or chronic. Acute sepsis mostly occurs from leakage of the ileo-anal anastomosis[119,120], which has been reported in 3-15% of patients. Pelvic sepsis is any inflammatory process within the pelvis due to infection and is not synonymous with anastomotic leakage.

Pouch leakage may be insidious, with case series reporting between 8 and 15% of patients with leaks describing very mild or absent symptoms, delaying the diagnosis. One of the arguments for not defunctioning pouches is that a leak will manifest sooner, leading to more expedient treatment and overall reduced inflammatory burden on the pouch. Fever and abdominal pain are the most likely presenting symptoms, followed by rectal pain, per-anal leakage and tenesmus [121–123].

The suspicion of pelvic sepsis should extend beyond the short-term post-operative period. In a series of patients referred to a tertiary centre for the investigation of antibiotic-dependant refractory idiopathic PIP, 38% were shown to have a pelvic collection[124].

1.10.3 Inflammatory complications of the pouch

1.10.3.1 Primary idiopathic pouchitis

PIP is a non-specific inflammatory condition in the ileal mucosa in the pouch[125]. It almost exclusively occurs in patients treated for UC and is rarely seen in FAP[126,127]. Its pathophysiology is poorly understood. The incidence of PIP is 20% at one year and up to 40% at 5 years[103]. Many of these patients have either a single episode of PIP which is short lived or recurrent acute attacks interspersed with periods without PIP. Despite treatments ten to 15% of patients with PIP experience chronic PIP[128,129] This is poorly defined but considered when patients fail to respond to

four weeks of antibiotic treatment. PIP is characterised by increased frequency of defaecation and fluidity of the stool sometimes with urgency and tenesmus, per anal bleeding, abdominal cramps, and is often associated with extra-intestinal manifestations[130]. Importantly, Moskowicz has shown that some degree of inflammation following ileostomy closure in present at 6 months and therefore suggested that the diagnosis of PIP should include diarrhoea, endoscopic inflammation and histological inflammation to include moderate or severe polymorph nuclear cell infiltration with ulceration in more than 25% of a low powered field[131].

1.10.3.2 Risk factors

Risk factors for PIP include, extensive UC[100,132,133] backwash ileitis before RPC [132], proctocolectomy thrombocytosis[134], concurrent primary sclerosing cholangitis[67,135,136], seropositive perinuclear antineutrophil cytoplasmic antibodies (pANCA),[137] being a non-smoker[133,138] and use of nonsteroidal anti-inflammatory drugs (NSAID)[133,138]. Other risk factors include genetic polymorphisms including the IL-1 receptor antagonist[139–141] , NOD2/CARD15 [142] and non-carrier status of TNF allele[141].

The treatment of acute PIP is largely empirical with antibiotics. Ciprofloxacin and metronidazole are most commonly used often with a rapid and dramatic response[143–146]. Ten to 15% of patients with PIP experience chronic PIP[128][129]. Various studies have demonstrated efficacy of treatments which include, antibiotics[147–149] ,steroids[150,151] and biologics[152–154].

1.10.3.3 Diagnosis

The diagnosis of PIP requires clinical, endoscopic and histopathological assessment. Currently there are no universally accepted criteria for the diagnosis but the 18-point pouch disease activity index is the most commonly used. It is made up of three domains that include clinical, histological and endoscopic data. The diagnosis requires essentially the presence of histologically proven acute inflammation in the ileal pouch mucosa.

1.10.3.4 Pre-pouch Ileitis

Pre-pouch ileitis (PPI) has no standard definition but is characterised by the presence of mucosal inflammation of the ileum immediately proximal to the pouch. Its estimated incidence is 6%[62,155]. The inflammation can extend for up to 50cm into the afferent limb [156] but this is unusual[62]. It occurs almost exclusively in patients who have had RPC for UC and is rarely seen in patients who have RPC for familial adenomatous polyposis (FAP)[156]. McLaughlin et al [62] showed that PPI was always associated with inflammation in the pouch (PIP). It has been suggested that the condition is indicative of CD and not UC[60,61], in studies in which CD was diagnosed by the presence of ulcerated lesions of the small bowel[60] or where the colectomy specimen had either non-necrotizing granulomas or transmural lymphoid aggregates in areas that were not deeply ulcerated[61]. More recent reports, however have suggested that PPI is not associated with CD but has histological features consistent with UC[62]. Data on the treatment of PPI are limited, with only one study suggesting that antibiotics may have benefit with 12/14 (86%) patients entering symptomatic remission following 28 days of Ciprofloxacin and Metronidazole[157].

1.10.3.5 Retained anorectal stump

Where there is an anastomosis between the pouch and the rectum, the term ‘cuffits’ has come to mean inflammation in the rectum between the anastomosis and the dentate line. It is seen on endoscopy and is confirmed by histology, with the mucosa of the pouch completely separate[158–160]. The incidence of inflammation of the retained rectal cuff stump has not been extensively studied with some reporting a 9% incidence[161]. The symptoms of distal mucosal inflammation are characterised by the frequent passage of stool with small quantities of blood[158]. Shen *et al*[159] reported 14 patients who were treated for inflammation of the retained rectal cuff using Mesalamine suppositories[159] whilst a benefit was shown, inflammation of the retained rectal cuff was poorly defined with improvement recorded on a invalidated scoring system. A small study also showed some success using endoscopic injection of the mucosa with long-acting steroids[162].

1.11 Mechanical issues with the pouch

1.11.1 Ileo-anal anastomotic stricture

The prevalence of stricture of the ileo-anal anastomosis has been reported to be as high as 38%[163]. Two locations are prone to develop stricture formation, the pouch-anal anastomosis (pouch outlet)[163] and the pouch inlet at the junction of neo-terminal ileum and pouch although other sites have been identified such as the site of where the stoma was reversed. Causes include fibrosis associated with a surgical complication at the anastomosis, CD, ischaemia, abscess and NSAID use.

Management of stricture includes dilatation either endoscopically or by bougie dilatation under general anaesthetic, [163] or self-dilatation at home if necessary[164]. In a series of 150 cases with stricture the 5, 10 and 25-year pouch retention rates were 97%, 90.6% and 85.9% in those patients undergoing balloon dilatation[165]. Risk factors for pouch failure included multiple strictures, underlying CD, surgery-associated strictures and malignancy[165]. Multiple stricturoplasties may be required with concomitant medical therapy to save the pouch. Surgical treatment includes defunctioning by proximal ileostomy, resection of the stricture and appropriate reconstruction or even excision of the pouch[163,164,166]. It has been suggested that smokers with UC strictures may have a worse outcome with balloon dilatation[167].

1.11.2 Dysplasia and cancer

1.11.2.1 Ulcerative colitis

In a pooled analysis of pouch cancers cumulative incidences of pouch-related adenocarcinoma after RPC were 0.12 (95% CI, 0.11–0.13), 0.19 (95% CI, 0.18–0.20), 0.29 (95% CI, 0.28–0.3), and 0.33% (95% CI, 0.31–0.34), 5, 10, 15, and 20 years, respectively. Primary pouch cancer cumulative incidences did not exceed 0.02% (95% CI, 0.01–0.12) 20 years after RPC[168].

A study of 3203 patients who had undergone RPC for IBD demonstrated a cumulative incidence for pouch and anal transition zone (ATZ) dysplasia at 5, 10, 15, 20 and 25 years of 0.8%, 1.3%, 1.5%, 2.2% and 3.2% respectively and a cumulative incidence of cancer (adenocarcinoma, squamous cell carcinoma and pouch lymphoma) of 0.2%, 0.4%, 0.8%, 2.4%, and 3.4%, respectively[35]. A cohort study of 3203 patients with a preoperative diagnosis of IBD, found that the risk of subsequent adenocarcinoma was

higher in patients who had a restorative proctocolectomy for colorectal cancer (6.8%) or dysplasia (2.7%) than those who had colectomy for refractory colitis (0.59%)[169].

1.11.2.2 Familial adenomatous polyposis (FAP)

Small amounts of rectal mucosa remain even after mucosectomy and adenomas have been reported in the anal canal in 10–30% of FAP patients following RPC with or without mucosectomy. The risk is about twice as high following a stapled anastomosis[70]. In addition, adenomas and even carcinomas can develop in the ileal mucosa of the pouch. In a study of 117 patients with a history of FAP and colectomy with RPC who were followed for a median 125 months, 30 dysplastic polyps were found (all low with grade dysplasia) and a single adenocarcinoma developed after 284 months. The median time to the development of dysplasia was 149 months (range, 15-405 months). The risk of dysplasia at 10, 20 and 25 years was 17, 45 and 69% respectively[95].

1.11.2.3 Risk factors

There is evidence demonstrating that a history of colorectal neoplasia[35,94,97,98,100] and IBD duration[72,94] are risk factors for the development of pouch dysplasia. Other risk factors for pouch dysplasia include the presence of severe chronic inflammation of the pouch[101,170] and severe acute inflammation histologically following RPC[171] and concurrent PSC[102]. A family history of colorectal cancer, chronic pouch inflammation, including chronic PIP and chronic refractory cuffitis have also been proposed as a risk for the development of dysplasia however the evidence for these are less clear[72]. A single case report highlighted that as early as 4 years after RPC after gradual development of severe atrophy in the ileal mucosa the patient developed low grade dysplasia[172]

1.11.2.4 Surveillance

In patients with UC there is not a strong evidence base to guide surveillance of the pouch mucosa. It should be reiterated that cancer in the pouch is a rare phenomenon and therefore unnecessary to offer surveillance to all patients. International guidance suggests that in patients without a history of neoplasia, a pouchoscopy should be performed annually [173].

In patients with FAP annual surveillance of the pouch has been recommended for five years and then at three yearly intervals in patients without adenomas in the pouch on earlier pouchoscopy[95].

1.12 Non-inflammatory complications of the pouch

1.12.1 Irritable pouch syndrome

Irritable pouch syndrome (IPS) is characterized by increased stool frequency, urgency and abdominal cramps as well as visceral hypersensitivity in the presence of normal pouch mucosa [108]. There is no formal definition and diagnosis which reflects the varied reported prevalence between 18%-43%[108,109,174] in studies.

IPS can be difficult to distinguish from other pouch pathologies. The diagnosis of IPS requires exclusion of other pathologies using a detailed history, examination, pouchoscopy and MRI [174].

Management is targeted at alleviating symptoms but is often ineffective[174]. Codeine and loperamide can be used to treat urgency and frequency of defaecation. Low dose amitryptylline can be used to relieve pain and associated mood disturbance. Referral to a dietician to try an exclusion diet (such as the low-FODMAP diet or wheat/dairy exclusion) may be beneficial. Eating an earlier evening meal may help reduce nocturnal frequency. Bio- feedback may help encourage a better bowel routine and help patients cope better with their symptoms. Appropriate counselling by the surgeon or gastroenterologist or an experienced pouch or stoma nurse before RPC is important to ensure that the patient is fully informed of the wide range of function that can be experienced following surgery[175]. This can help to avoid unrealistic expectations.

1.12.2 Weak sphincter

A weak sphincter may result from damage during the operation or due to poorly selected patients prior to RPC. Reports have also suggested that pregnant patients with RPC can have sphincter damage through natural delivery[176]. Treatment is difficult but evidence suggests biofeedback may be of benefit in these patients[177]

1.12.3 Bile salt malabsorption

Bile salt malabsorption can result in a watery diarrhoea Bile salts are important in fat soluble vitamin absorption. Bile salt reabsorption is impaired usually due to ileal disease or resection. There have also been reports of fat malabsorption, which appears to be associated with bile acid absorption and was found to be reduced in RPC patients compared to patients who underwent ileostomy[178]. In patients with clinical pouch dysfunction, presumed faecal stasis with bacterial overload could lead to bile salt deconjugation and decreased absorption, as measured by 75Se homotaurocholate, compared to patients with healthy pouches[178]. Treatment includes bile acid sequestrants.

1.13 Pouch salvage surgery

Pouch salvage surgery includes pouch revision and redo pouch surgery. Fonkalstrud and Burstorff Silva[179] reported good long-term results (mean 7.7 years) with improvement in symptoms occurred in 98% of transanal revisions, 91.5% of AP reconstructions, 86% of new pouch constructions, and 100% of conversions of a straight pull-through to a pouch[179]. Tekkis *et al*[180] reported on their experience of abdominal salvage surgery, after a mean follow-up of 46 (range 1-147) months. During this follow-up period twenty-four patients (21.4 per cent) experienced pouch failure, the incidence of which increased with time. The pouch failed in all patients with CD. Successful salvage at 5 years was significantly associated with non-septic (85 per cent) rather than septic (61 per cent) indications (p= 0.016). Frequency of night-time defaecation and faecal urgency improved after salvage surgery (p = 0.036 and p = 0.016 respectively at 5-year follow-up; n = 32). Remzi et al[181] reported on 502 (43% males) patients with a median age of 38 years who underwent restoration surgery. A new pouch was created in 41% of patients whereas 59% had their original pouch revised and retained. They reported a postoperative mortality of 0% and morbidity was 53%. The short-term anastomotic leak rate was 8%. At a median follow-up of 7 years after redo surgery, 101 (n=20%) patients had pouch failure. Pelvic sepsis developing after redo ileoanal pouch surgery was the primary indicator of pouch failure (hazard ratio, 3.691; 95% confidence interval, 2.411-5.699; P<0.0001). They also reported overall functional outcomes and QOL scores were acceptable.

1.14 Aims of thesis

This thesis will explore the inflammatory pouch complications highlighted in the introduction. I will undertake a systematic review with meta-analysis to explore what is currently known about the treatment of chronic PIP. I will then understand how effective antibiotics and biologics are at achieving remission in a cohort of patients with chronic pouchitis and pre-pouch ileitis. I will also review the effectiveness of non-medical therapies such as biofeedback and the Renew® anal insert in aiding patients with pouch pathologies. The second part of my thesis aims to try and understand some of the mechanisms that may contribute to inflammatory pouch pathologies through 16s microbiota analysis and metabonomic profiling.

Chapter 2

Inflammatory problems associated with the pouch

The most common medical complications of the pouch are inflammatory in nature. This chapter will discuss the incidence and management of inflammatory pouch problems to include PIP and pre-pouch ileitis. To understand how best to manage inflammatory pouch pathologies I undertook a systematic review which explored the management of both acute and chronic pouchitis to culminate in an evidenced based treatment algorithm.

2.1 Systematic review with meta-analysis - management of chronic PIP with an evidence-based treatment algorithm

2.1.1 Introduction

2.1.1.1 Acute primary idiopathic pouchitis

The incidence of acute PIP is 20% at one year and up to 40% at five years[103]. PIP is clinically characterized by variable symptoms including increased stool frequency and fluidity, per anal bleeding, abdominal cramping, urgency and tenesmus, incontinence, fever and extraintestinal manifestations[130].

Acute PIP is defined by inflammation of the ileoanal pouch that usually resolves with a 4-week treatment and reoccurs less than three times in a year. To date there have been four randomised controlled trials on the management of acute PIP. Shen *et al* showed that both Ciprofloxacin and Metronidazole had similar efficacy and significantly improved symptoms based on the PDAI score[145]. Sambuelli *et al* showed that a Budesonide enema had similar efficacy to metronidazole based on the PDAI score[182]. Kuisma *et al* demonstrated that the probiotic Lactobacillus rhamnosus GG was ineffective at significantly reducing PDAI when compared with placebo. Issacs *et al* showed that Rifaximin lowered the PDAI score but did not reach statistical significance when compared with placebo[183].

There have also been a number of non-randomised studies. One study utilised a probiotic using 500 ml of a fermented milk product (Cultura) containing live lactobacilli (La-5) and bifidobacteriae (Bb-12) was given daily for 4 weeks to patients with PIP and highlighted significant reductions in involuntary defecation, leakage, abdominal cramps, faecal frequency and sensation of urgency[184]. Gionchetti *et al* also showed that the probiotic VSL#3 significantly improved quality of life in patients given a four week trial of VSL#3[185]. Despite the limited data antibiotics remain the mainstay of treatment for acute PIP.

2.1.1.2 Chronic PIP

Despite best treatment 10-15% of patients with acute PIP will develop chronic PIP. The following chapter will explore the treatment options for patients with PIP. A systematic review was conducted looking at the treatments of chronic PIP.

A systematic review with meta-analysis in 2010 reviewed the efficacy of antibiotics and probiotics in PIP[186]. A systematic review in 2015 explored the use of biologics in PIP[187]. A further meta-analysis in 2014 reviewed the role of probiotics with the focus on maintenance of remission[188]. A Cochrane review in 2015 appraised two randomised controlled trials in the treatment and prevention of chronic refractory PIP[189]. This systematic review with meta-analysis builds on these reviews, adding information from all studies that treated chronic refractory PIP. Using medical databases and other sources, I reviewed the latest evidence in treating chronic refractory PIP. In addition to antibiotics, there is evidence that steroids, immunomodulators and biologics all have a role in treating chronic PIP.

2.1.2 Objectives

To determine the efficacy of oral and topical medical therapies including antibiotics, probiotics, immunomodulators, steroids and biologics for the treatment of chronic refractory PIP in patients who have undergone RPC for UC.

2.1.3 Methods

2.1.3.1 Types of studies

Randomized controlled trials, cohort studies, observational studies and case reports were considered. Studies which reported duplicate results were excluded. Those where data could not be extracted were also excluded.

2.1.3.2 Types of participants

Adult patients (age ≥ 18 years) with chronic refractory PIP were included. Chronic refractory PIP was defined by each study. For the purpose of analysis, I used each study's definition of chronic refractory PIP for the systematic review. I excluded studies that only reported on acute PIP.

2.1.3.3 Types of outcome measures

The primary outcome was the proportion of patients with clinical improvement or remission of PIP. The definition of clinical improvement or remission varied from study to study, meaning that it was difficult to make comparisons across studies. The definitions of clinical improvement or remission used in each study was used for extraction of the data.

2.1.3.4 Search methods for Identification of studies

A computer assisted search of the on-line bibliographic database MEDLINE and EMBASE was carried out between 1966 and February 2016 by two independent researchers (JPS and NSD). The following medical Subject Heading (MeSH) terms were used which included both the root term and text words. Synonyms and word variations were combined using the "OR" function and then combined with other key terms using the "AND" function: "refractory" "chronic", "long term", "difficult", "unmanageable", "ulcerative colitis", "UC", "colitis", "ileum", "ileostomy", "postoperative complications", "PIP", "colonic pouches", "pouch", "proctocolectomy"," restorative", "colitis", "RPC","RPC", "j-pouch", "s-pouch"," w-pouch", "treatment", "management", "medication", "therapy", "therapeutics", " anti-TNF", "antibiotics", "steroids", "tumour necrosis factor-alpha", "remission", "spontaneous", "remission induction", "resolution", "cure"{|CL4|}.{|CL4|{|CL5|}}{|CL5|} Manual searches of the reference list from the potentially relevant studies were performed in order to identify additional studies that

may have been missed using the computer-assisted search strategy. Abstracts from conferences from the American Gastroenterological Association, American Society of Colon and Rectal Surgery, European Crohn's and Colitis, United European Gastroenterology and the British Society of Gastroenterology were also manually searched from 1965-2016 in order to identify unpublished studies. I did not restrict the search to articles in English language.

2.1.3.5 Data collection and analysis

2.1.3.5.1 *Study selection*

Potentially relevant articles were reviewed in an independent fashion by two authors (JPS and Nik Sheng Ding (NSD)) to determine whether they met the inclusion criteria. The studies were then labelled as eligible, ineligible, or having insufficient information to make a judgement as to eligibility (which were then excluded). Any discrepancies were addressed by a joint re-evaluation of the original article.

2.1.3.5.2 *Data Collection*

Eligible articles were reviewed by JPS and NSD and the results from the included articles were extracted into tables. The proportions of patients who had clinical improvement or entered remission were derived from each study.

2.1.3.6 Risk of bias

Two authors (JPS and NSD) independently assessed the methodologies using the Cochrane risk of bias tool for randomised controlled trials as described in the Cochrane handbook for systematic reviews of interventions[190]. Assessment of bias was judged as "yes": low risk of bias, "No": high risk of bias, or "unclear" unknown risk of bias. The Risk Of Bias In Non-randomized Studies – of Interventions (ROBINS-I) assessment tool was used to assess bias in non-randomised controlled studies[191]. Assessment of bias was judged as low bias, moderate bias, serious bias, critical bias or no information. Disagreements were resolved by consensus. (figure 2).

2.1.3.7 Statistical analysis

For analysis the outcome of remission was considered as a binary outcome (yes/no). Meta-analysis methods were used to pool the percentage of patients in remission from

the various studies. The analysis was implemented using the ‘metaan’ package with Stata.

For each study, the standard error of the proportion in remission was calculated using the normal approximation to the binomial distribution. For studies where the outcome was not observed in any patients, or in all patients (i.e. a 0% or 100% remission occurrence), the standard error was approximated by half the width of 95% confidence calculated using the exact binomial method.

The heterogeneity between studies was assessed based on the significance of the between-study heterogeneity, and also on the size of the I^2 value. Substantial heterogeneity was assumed if the I^2 value was above 50%. If there was substantial heterogeneity between studies, studies were pooled using the DerSimonian-Laird random-effects method. A random effects model was also used if there was no heterogeneity between studies. The analyses were performed for all studies combined, and then separately for each type of medication (figure 2).

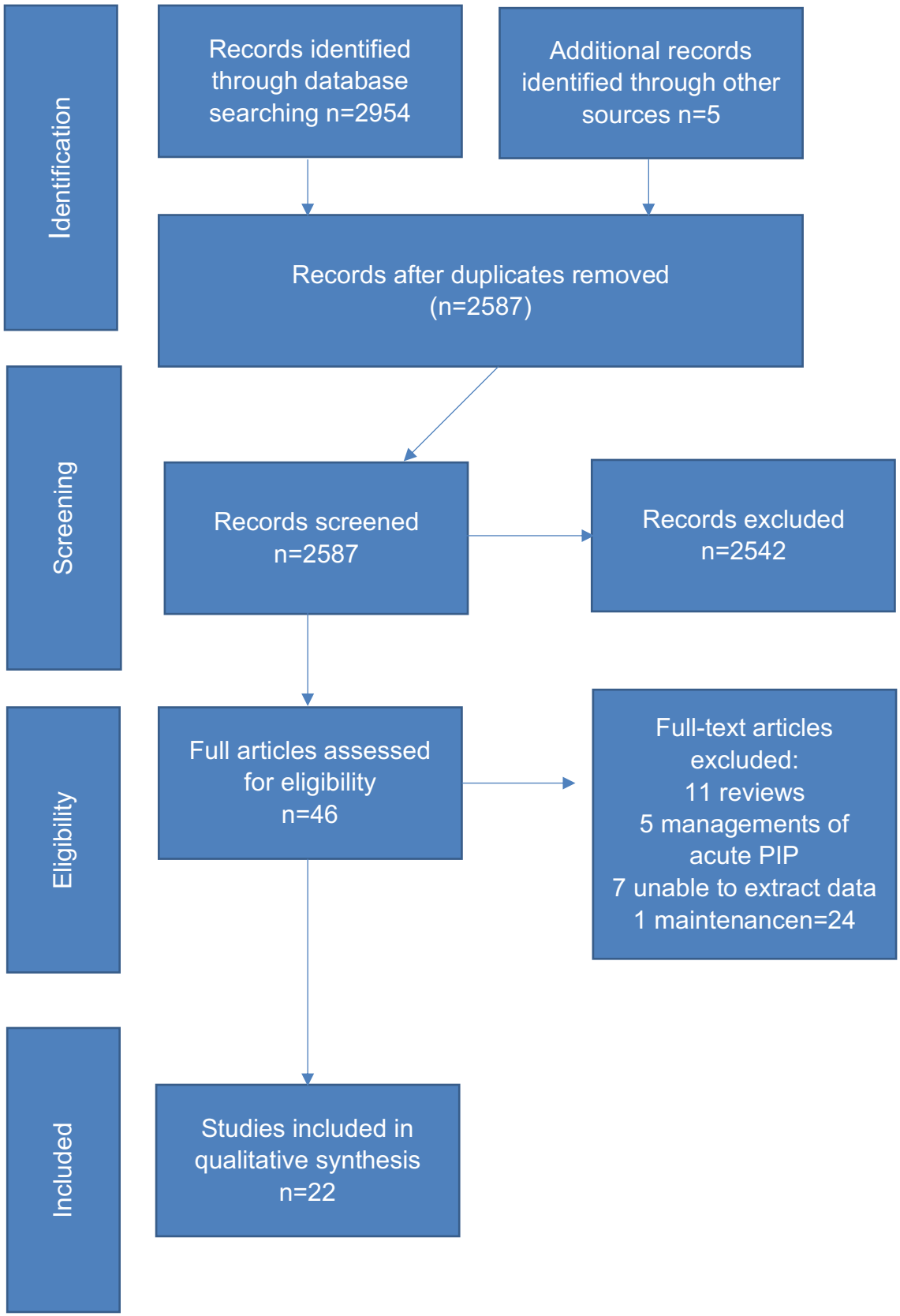

Figure 2. Prisma diagram demonstrating how inclusion of studies were derived

2.1.4 Results

2.1.4.1 Description of studies

The literature search identified a total of 2954 studies. After removing duplicates 2587 studies remained for review of title and abstracts for eligibility. Two authors (JPS and NSD) independently reviewed the titles and abstracts of these studies. After screening abstracts 45 articles were reviewed in full. After screening individual papers 16 were included in the study. Six additional papers were included after manual reference searching. Therefore, a total of 22 papers were considered eligible.

2.1.4.2 Variable definition of chronic PIP

There was some variation in the definition of chronic refractory PIP used within each study. The majority of studies, 16/21 (76%) defined chronic PIP as greater than four weeks of symptoms despite having used antibiotics or alternative standard therapies. Three studies defined chronic PIP as requiring continuous antibiotics. In two studies we did not categorise the definition of chronic PIP.

2.1.4.3 Table 1. Summary of treatment interventions

Table 1. Summary of treatment interventions

Study	Year	Intervention	Study Design	n	Summary Outcomes	Assessment of Bias
Antibiotics						
Madden[3]	1994	Metronidazole 400mg vs Placebo	RCT	11	8/11 (73%) improved in stool frequency in antibiotic group 0/11 (0%) improvement of stool frequency in placebo group	low
Gionchetti[4]	1999	Rifaximin 1g BD and Ciprofloxacin 500mg BD for 15 days	Observational	18	6/18 (33%) achieved remission	low
Mimura[6]	2002	Metronidazole (400mg or 500mg) BD and Ciprofloxacin 500mg BD for 28 days	Observational	42	36/42 (82%) achieved remission	low
Abdelrazek [9]	2005	Rifaximin and 1g BD and ciprofloxacin 500mg BD for 14 days	Observational	8	7/8(88%) achieved remission	low
Shen[16]	2007	Ciprofloxacin 1g/day and Tinidazole 15mg/kg for 4 weeks	Observational	16	14/16 (88%) achieved remission	low
Steroids						
Gionchetti[17]	2007	Budesonide 9mg/day for 8 weeks	Observational	20	15/20 (75%) achieved remission	low
Gionchetti[18]	2014	Beclomethasone Dipropionate 10mg/day for 8 weeks	Observational	10	8/10 (80%) achieved remission	low
Biologics						

Study	Year	Intervention	Study Design	n	Summary Outcomes	Assessment of Bias
Viscido[31]	2004	Infliximab 5mg/kg (week 0,2,6 then every 8 weeks for a year)	Observational	7	6/7 (86%) achieved remission	low
Gionchetti[24]	2010	Infliximab 5mg/kg (week 0,2,6) for 10 weeks or Adalimumab 160/80mg induction then 40mg alternate weeks	Observational	12	9/12 (75%) achieved remission in infliximab group 5/7 (72%) achieved remission in adalimumab group	low
Ferrante[23]	2010	Infliximab 5mg/kg (week 0,2,6) for 10 weeks	Observational	11	3/11 (27%) achieved remission	low
Viazis[25]	2012	Infliximab 5mg/kg (week 0,2,6 then every eight weeks for a year)	Observational	7	5/7 (72%) achieved remission	low
Barreiro-de Acosta[26]	2012	Infliximab 5mg/kg (week 0,2,6) Followed by 5mg/kg every eight weeks or 10mg/kg every 10 weeks based on clinical need	Observational	33	7/33 (21%) achieved remission at week eight 11/33(34%) achieved remission at 26 weeks 9/33 (27%) achieved remission at 52 weeks	low
Barreiro-de Acosta[27]	2012	Adalimumab 160/80mg induction followed by 40mg alternate weeks for 26 weeks	Observational	8	1/8 (13%) achieved remission at eight weeks 1/8 (13%) achieved remission at 26 weeks	low
Lizuka[32]	2014	Infliximab 5mg/kg (week 0,2,6 then every eight weeks for a year)	Observational	1	1/1 (100%) achieved remission	low

Study	Year	Intervention	Study Design	n	Summary Outcomes	Assessment of Bias
Bismuth						
Tremaine[19]	1997	Bismuth Carbomer enema 270mg vs placebo	RCT	20	0/20 (0%) achieved remission in bismuth group 0/20 (0%) achieved remission in placebo group	low
Gionchetti[20]	1997	Bismuth Carbomer enema at night for 45 days	Observational	12	10/12 (83%) achieved remission	low
Alicaforsen						
Milner[21]	2004	Alicaforsen 240mg enema at night for six weeks	Observational	12	7/12 (58%) achieved remission	low
Tacrolimus						
Ng[30]	2006	Tacrolimus 0.1mg/kg/day to reach a trough level of 5-10ng/ml	Observational	1	1/1 (100%) achieved remission	Serious
Uchino[22]	2013	Tacrolimus enema 0.08mg/kg every morning for 8 weeks	Observational	10	7/10 (70%) achieved remission	low
FMT						
Landy[28]	2013	30 g of fresh donor stool via nasogastric tube	Observational	8	0/8 (0%) achieved remission	low
Elemental Diet						
McLaughlin[29]	2013	Elemental diet for 28 days	Observational	7	5/7 (71%) reported a reduction in stool frequency	low

2.1.4.4 Antibiotics

Gionchetti *et al*, 1999[147] conducted a cohort study of 18 patients with chronic PIP who were treated orally with rifaximin 1g BD and ciprofloxacin 500mg BD for 15 days. PIP was defined as a pouch disease activity index (PDAI) ≥ 7. Chronic PIP was defined as no response after treatment with antibiotics (such as metronidazole, or ciprofloxacin or amoxycillin/clavulanic acid) for at least 4 weeks. Six out of 18 (33%) patients went into remission and 10/18 (56%) improved after 15 days. The median PDAI scores before and after therapy were 11 (range 9–17) and 4 (range 0–16), respectively ($p < 0.002$). No adverse events were reported.

Abdelrazek *et al* 2005[148] conducted a cohort study on eight patients with chronic PIP who were treated with two weeks of rifaximin 1g BD and ciprofloxacin 500mg BD. PIP was defined using the PDAI. Chronic PIP was defined as no response to at least four weeks of standard antibiotic therapy or relapse immediately when antibiotic treatment was stopped or reduced. Remission was defined as an improvement of three points on the PDAI. Seven of the eight (88%) patients either went into remission ($n = 5$) or improved ($n = 2$). The median (range) PDAI scores before and after therapy were 12 (9–18) and 0 (0–15), respectively, ($P = 0.018$). There were no significant side effects reported.

Shen *et al*, 2007[149] conducted a cohort study of 16 consecutive patients with chronic PIP who were treated with a four week course of ciprofloxacin 1g/day and tinidazole 15mg/kg/day. A historic cohort of 10 consecutive patients with chronic PIP treated with oral mesalamine (4g/day) or enema (8g/day) or suppository (1g/day) were used as controls. All patients had a PDAI ≥ 7 at entry. Chronic refractory PIP was defined as symptoms for more than four weeks with endoscopic and histological inflammation despite treatment with single or dual antibiotics for more than four weeks. In the antibiotic group, 87.5% of patients achieved clinical remission and 88% achieved clinical response, compared to 50% in the mesalamine group for remission and 50% for response. This was however not statistically significant ($p=0.069$). In the antibiotic group, two patients had adverse events (peripheral neuropathy and dysgeusia) but continued treatment.

2.1.4.5 Steroids

Gionchetti et al, 2007[192] conducted an open-label non-randomised study in 20 consecutive patients with chronic PIP who were treated with budesonide controlled ileal release 9mg/day for eight weeks. Chronic PIP was defined as a total PDAI score of ≥ 7, and not responsive to antibiotics for four weeks. Remission was defined as a combination of PDAI of ≤ 2, endoscopic score ≤ 1 and total PDAI score ≤ 4. Fifteen of 20 patients (75%) achieved remission. The median total PDAI scores before and after therapy were, respectively, 14 (range 9-16) and 3 (range 2-10) ($P < 0.001$).

Gionchetti *et al*, 2014[151] conducted an open-label non-randomised study in 10 consecutive patients with chronic PIP who were treated with beclomethasone 10mg/day for eight weeks. Current active refractory PIP was defined as a PDAI score of ≥ 7 and no response to at least four weeks of standard antibiotic treatment (ciprofloxacin 1g once a day or metronidazole 1g once a day). Remission was defined as a combination of PDAI clinical score of ≤ 2, endoscopic score of ≤ 1 and a total PDAI score of ≤4. Eight of the 10 (80%) treated patients achieved remission, while two had only a mild improvement. The median bowel frequency decreased significantly from 10 (range 7–15) to six (range 3–11) after steroid treatment ($p < 0.001$).

2.1.4.6 Enemas

Tremaine *et al*, 1997[193] conducted a randomised, double-blind placebo controlled trial in 40 patients with chronic PIP who were randomly assigned either 270mg bismuth enema (n=20) or placebo (n=20). Chronic PIP was defined as continuous symptoms of PIP for more than four weeks and a PDAI score ≥7. Patients had either failed or were intolerant to metronidazole as well as other commonly used treatments for PIP. Remission was defined as a reduction in the PDAI by at least three points at three weeks. There were no significant differences between the populations at baseline. At week three, 9/20 (45%) of patients in both the bismuth and placebo groups had improved. No patient achieved remission in either group. There was no significant difference in response to therapy in the treatment or the placebo with regard to remission. One patient in the bismuth group reported a worsening of diarrhoea requiring hospital admission.

Gionchetti *et al*, 1997[194] conducted an open label non-randomized study in twelve patients with chronic PIP who were administered bismuth carbomer enema at night for 45 days. Chronic PIP was defined as continuous symptoms for more than four weeks and the need for antibiotics or steroids for more than 15 days per month to control symptoms. Clinical remission was defined as a decrease in PDAI ≥2. Ten of 12 treated patients (83%) went into remission after 45 days. No serious side effects were reported.

Milner *et al*, 2004[195] conducted an open-label, uncontrolled study in 12 patients with chronic PIP who were treated with 240mg alicaforsen antisense enema nightly for six weeks. Patients underwent two weeks of washout prior to enrolment. Chronic refractory PIP was defined as patients who had symptoms for greater than four weeks and had failed alternative therapies, with a PDAI score of ≥ 7. The primary endpoint was a reduction in PDAI from baseline at week six. At week six, 7/12 (58%) of patients were in remission with PDAI < 7. The mean decrease in PDAI from baseline was six points. No drug related serious or significant adverse effects were reported during the study.

Uchino *et al*, 2013[196] conducted a non-randomized open-label study in 10 patients with chronic refractory PIP who were treated with once daily tacrolimus enema ($0.08 mg kg^{-1}$) in the morning for eight weeks. Chronic PIP was defined by no response to a four-week course of a single antibiotic (metronidazole or ciprofloxacin) and requiring therapy of for at least four weeks of dual antibiotics. A PDAI ≥7 was used as confirmation of the diagnosis. Clinical remission and clinical response were defined a clinical sub-score of zero points and a clinical sub-score decrease of more than three points. Seven of the 10 patients achieved complete remission of clinical symptoms, and a total of nine patients were clinical responders. The mean PDAI score decreased significantly to 7.8 ± 0.8 points (range, 6–15) after eight weeks ($p < 0.01$). Three patients reported feeling mild burning in the pouch, which was not sufficient to warrant discontinuation of the eight-week application.

2.1.4.7 Biologics

Ferrante *et al*, 2010[152] conducted a retrospective study in 11 patients with chronic refractory PIP who were treated with standard infusions of infliximab (5 mg/kg body

weight). Chronic refractory PIP was defined as symptom duration greater than four weeks following standard treatment. A complete clinical response was defined as cessation of diarrhoea, urgency, incontinence, blood loss and abdominal pain. A partial clinical response was defined as a marked clinical improvement, but persisting symptoms. All other outcomes were defined as no short-term clinical response. Long-term response was evaluated at last follow-up. Short-term response to infliximab was evaluated at week 10. At week 10, 1/11 (9%) patients did not show any clinical benefit and needed a permanent ileostomy, 7/11 (64%) patients had a partial clinical response, and 3/11 (27%) had a full clinical response. The modified PDAI (mPDAI) dropped significantly from nine to five ($p = 0.011$). In the subgroup of 10 patients with chronic refractory PIP in the absence of pouch fistula or prepouch ileitis who initially responded to infliximab, seven were still on infliximab after a median follow-up of 8.5 (range 2–38) months. Two patients had to stop because of a delayed hypersensitivity reaction, while one patient could be bridged to azathioprine. The remaining seven patients underwent a new endoscopy at the end of follow-up. Four of them did not show any lesion, while three had clear endoscopic activity despite a sustained clinical benefit.

Gionchetti *et al*, 2010[197] conducted an open-label non-randomised study in 19 patients with chronic PIP who were treated with either 5mg/kg of infliximab at weeks zero, two, six or adalimumab 160/80mg at weeks zero and two, then 40mg every other week. Chronic PIP was defined as unresponsive to a month of antibiotics or two months of budesonide. Remission was defined as a PDAI score of one. Short term efficacy was measured at week 10. Twelve patients received infliximab and five adalimumab. Nine of 12 (75 %) and 5/7 (71%) showed remission respectively in the infliximab and adalimumab group. The median PDAI scores before and after therapy were 13 (range 8-18) and 2 (range 0-9) in the infliximab group($p<0.001$), and 14 (range 9-18) and 2 (range 0-10) in the adalimumab group ($p<0.001$). No serious side effects were registered.

Viazis *et al*, 2011[153] conducted an open prospective cohort study in seven patients with chronic refractory PIP who were treated with infliximab 5mg/kg at zero, two, and six weeks and then, every two months for a year. Chronic PIP was defined as no response to at least four weeks of standard antibiotic therapy (ciprofloxacin 1g BD or

metronidazole 500 mg TDS). PIP was defined as a total PDAI score ≥ 7 points. Complete clinical response was defined as cessation of diarrhoea, urgency, incontinence, blood loss and abdominal pain. A partial clinical response was defined as a marked clinical improvement, but with persisting symptoms. All other outcomes were defined as no response. After one year of infliximab administration, 5/7(71%) patients had a complete clinical response, 1/7 (14%) had partial response (14%) and 1/7(14%) had no response. There were no major complications from infliximab administration, apart from a minor rash seen in one of the patients. The rash appeared at the beginning of the second infusion and disappeared after reduction in the rate of the infusion.

Acosta *et al*, 2012[198] conducted a retrospective open-label multicentre study on 33 patients with chronic PIP who were treated with 5mg/kg of infliximab with an induction regime (infliximab at weeks zero, two, and six) at doses of 5mg/body weight and 25 (76%) continued with a maintenance scheme (infliximab every eight weeks). Among these 25 patients, nine (36%) needed dose escalation (five of them to 10mg/kg and the other four to shorter time intervals between infusions). Chronic PIP included all patients with clinical and endoscopic findings of PIP who had previously failed antibiotics for at least four consecutive weeks and probiotics or immunosuppressive drugs. Short-term infliximab efficacy was evaluated at week eight and mid-term efficacy at weeks 26 and 52. Complete response was defined as cessation of diarrhoea and urgency and partial response as marked clinical improvement but persisting symptoms. Median time of infliximab follow-up was 60 weeks. At week eight, seven patients (21%) achieved complete response and 21 (63%) showed partial clinical response. Only five of the patients (15%) did not show any response. At week 26, after an intention to treat (ITT) analysis, 11 patients (33%) were in complete response and another 11 (33%) had shown partial clinical response. After analysing the patients who continued treatment at week 26, a complete response rate of 44% and a similar partial response rate of 44% were observed. At week 52, after an ITT analysis, nine patients (27%) were in complete clinical remission and another six (18%) had shown partial clinical response. After analysing the patients who continued at week 52 with treatment, an observed remission rate of 56% and a response rate of 38% was found. Thirteen patients (39%) had to withdraw infliximab treatment; five (15%) due to severe adverse events, (one lupus like reaction, four infusion reactions),

four (12%) lost response to infliximab during the trial period and four (12%) were primary non-responders.

Acosta *et al*, 2012[199]conducted a retrospective open-label study on eight patients with chronic PIP who had previously failed infliximab. Patients were treated with adalimumab 160/40mg as induction, followed by 40mg every alternate week. Chronic refractory PIP was defined by both clinical and endoscopic features of PIP that had failed to show a response to at least four weeks of antibiotics. Complete clinical remission was defined as cessation of diarrhoea, urgency and per anal bleeding. A partial response was defined as marked clinical improvement, but persistence of symptoms. Outcomes were measured at weeks 8,26 and 52. At week eight 1/8 (13%) achieved remission and 5/8 (63%) showed a clinical response. At week 26 following an ITT analysis 1/8 (13%) was in complete remission and 3/8 (38%) showed a clinical response. At week 52 after an intention-to-treat analysis 2/8 (25%) were in clinical remission and 2/8 (25%) showed a clinical response. There were no significant adverse events reported.

2.1.4.8 Other treatments

Landy *et al*, [200,201] conducted a non-randomised study in eight patients with chronic PIP who were given a 30g fresh donor stool via a nasogastric tube on a single occasion. Chronic PIP was defined as patients with PDAI≥7 who had not responded to standardized therapy. The outcome measure was remission at four weeks after faecal microbiota transplantation (FMT). At four weeks post FMT, no patient achieved remission however, two patients regained sensitivity to ciprofloxacin. Importantly, bowel lavage was not used in this study which may account for the negative results. No adverse events were reported.

McLaughlin *et al* 2013[202] conducted a non-randomized prospective study in seven patients who received 28 days of exclusive elemental diet enough to reach their daily energy requirements. Chronic PIP was defined as patients with a PDAI ≥7 who were unresponsive to four weeks of combined antibiotic treatment. Outcome was reduction in stool frequency at day 28 and reduction in PDAI. Treatment with elemental diet resulted in a significant reduction in stool frequency (from median 12 to 6, $p = 0.028$) and the PDAI symptom score (from 4 to 1, $p = 0.039$). There was a non-significant

trend towards an improvement in the ability to defer defecation (from 25 to 60 min, p = 0.078). There were no adverse events reported.

2.1.4.9 Interventions when chronic PIP was defined as requiring continuous antibiotics

2.1.4.9.1 *Antibiotics*

Mimura *et al*, 2002[146] conducted a cohort study of 44 patients with chronic PIP who were treated using a combination of metronidazole 400mg (UK population) or 500mg (Italian population) twice daily and ciprofloxacin twice daily for 28 days. Chronic PIP was defined as a history of PIP at least twice in the last 12 months or persistent PIP requiring continual antibiotics and a PDAI $\geq$ 7. Thirty six of 44 patients (82%) achieved remission. One patient withdrew from the trial as they developed nausea and dysgeusia to metronidazole.

Biologics

Viscido *et al*, 2004[203] conducted an open-label non-randomised study in a subgroup of seven patients with chronic PIP who were treated with 5mg/kg of infliximab at week zero, two and six. Treatment after this was “on demand” only. Patients also received 2.5mg/kg of azathioprine at the time of the first infliximab infusion. Chronic refractory PIP was defined as persistent active PIP unresponsive to continuous antibiotics. Complete response was defined as improvement in well-being and cessation of diarrhoea, urgency/incontinence, stool blood, abdominal pain. A partial response was defined as an improvement or reduction of the symptoms. All other outcomes were defined as no response. Among the seven patients with PIP who received infliximab, six had a complete clinical response, and one patient had partial clinical response 10 weeks after the first infusion. At six-month follow-up, one patient had developed a thoracic herpes simplex virus infection, which required treatment with acyclovir (4 g/day for 10 days), without withdrawal of immunosuppressive treatment.

Lizuka *et al*, 2014[204] reported a case of a 29-year woman with chronic PIP who was treated with infliximab at weeks zero, two, six and then every eight weeks up until a year. Chronic refractory PIP was defined as a PDAI of >10 after two years of antibiotic treatment. After 40 weeks of treatment the patient’s abdominal pain and clinical

symptoms subsided and her PDAI was five. She continued treatment for a year and remained in remission.

2.1.4.10 Effects of interventions when chronic PIP definition cannot be categorised

2.1.4.10.1 *Antibiotics*

Madden *et al*, 1994[143] conducted a double-blind crossover trial in 13 patients with chronic PIP who were treated with metronidazole 400mg or placebo. Patients were randomised to receive either metronidazole 400mg by mouth three times a day or placebo for two weeks. The drug was stopped for a wash out period. Remission was not defined but improvement in stool frequency was used as an assessment of improvement. There were 11/13 subjects who completed the trial; one withdrew due to an episode of intestinal obstruction. Stool frequency improved in 8/11 (73%) receiving metronidazole, worsened in two and was unchanged in one. Placebo had no effect on stool frequency in the 11 patients who received treatment. Metronidazole improved stool frequency by four actions/day ($p<0.05$ 95% CI). Six patients (55%) reported side effects whilst on metronidazole including an unpleasant taste (2), nausea (2), vomiting (1), abdominal discomfort (1), headache (1), skin rash (1).

2.1.4.10.2 *Tacrolimus*

Ng *et al*, 2006[205] conducted a retrospective single centre review of all patients with inflammatory bowel disease that received tacrolimus 0.05mg/kg twice daily orally. A sub group of patients with chronic PIP were included. Chronic PIP was defined as patients experiencing moderate to severe chronic active disease, were steroid dependent or had failed conventional therapy (azathioprine, 6-mercaptopurine or infliximab). Clinical remission was defined as a modified PDAI <5 at week four of treatment. All patients received an initial dose of 0.1mg/kg/day in two divided doses then dose was adjusted to reach a trough level of 5-10 ng/ml. One patient with chronic PIP took part in the study and achieved remission with a reduction of mPDAI from eight to four and a reduction of stool frequency from 25 to 12 times per day. There were no reported adverse events in this patient.

The pooled results for all studies, and for each medication separately, are summarised in the next table. These show the number of studies, and details of the heterogeneity

both in terms of the significance and the I^2 value. The pooled results are also shown, and are the pooled percentage in remission, along with corresponding confidence intervals.

Table 2. Pooled analysis of effectiveness of all medications.

Medication	Number of studies	Heterogeneity		Pooled % (95% CI)
		p-value	I^2	
All combined	22	<0.001	88%	60% (45%, 74%)
Antibiotics	5	<0.001	80%	74% (56%, 93%)
Steroids	2	0.75	0%	77% (62%, 92%)
Biologics	8	<0.001	83%	76% (53%, 76%)
Bismuth	2	<0.001	97%	41% (0%, 100%)
Alicaforsen	1	-	-	58% (28%, 85%)
Tacrolimus	2	0.57	0%	72% (45%, 100%)
FMT	1	-	-	0% (0%, 37%)
Elemental diet	1	-	-	71% (29%, 96%)

Graphical illustrations of the results for the individual studies are shown in the subsequent Forrest plots.

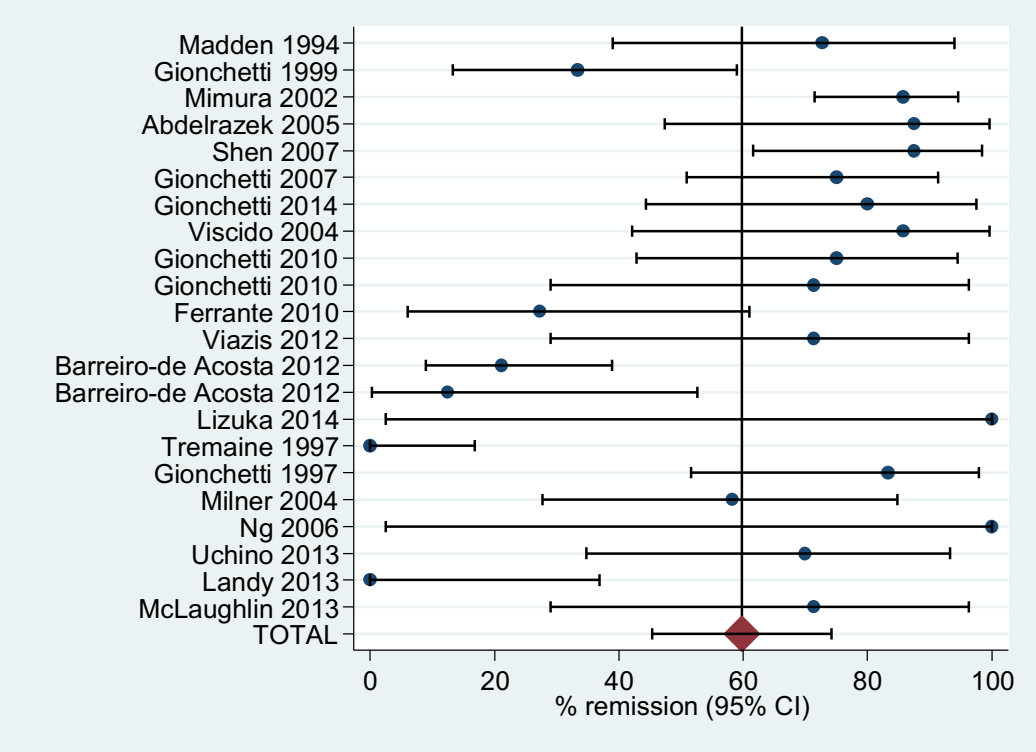

Figure 3. Results for all therapies for chronic PIP.

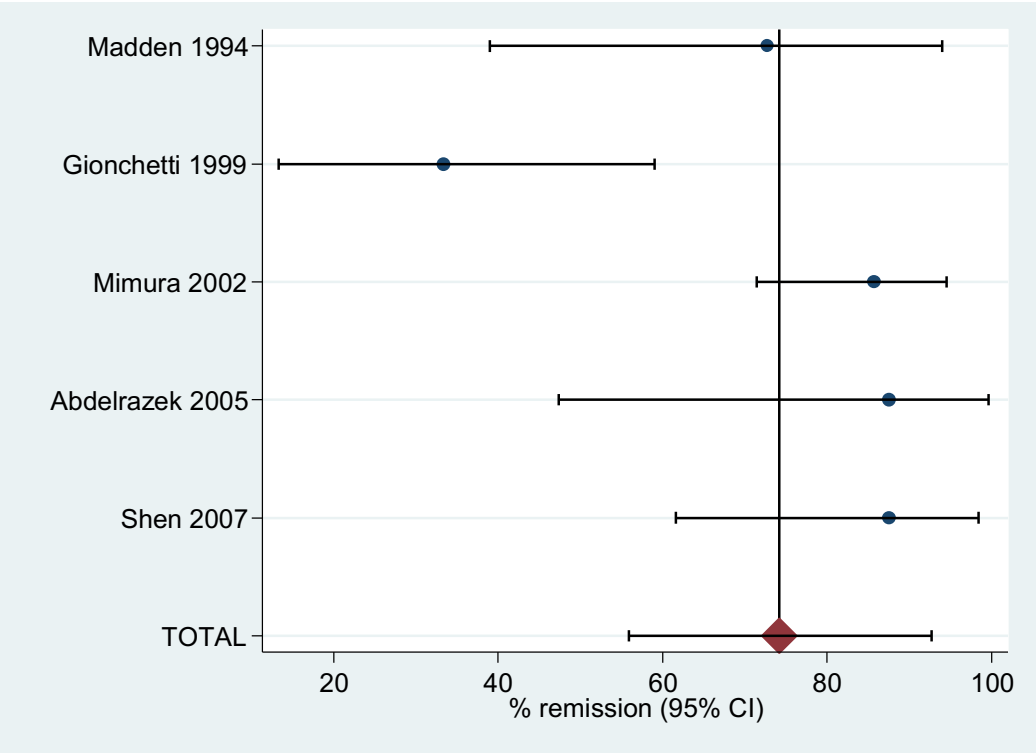

Figure 4. Results for antibiotics for chronic PIP.

Figure 5. Results for biologics for chronic PIP.

2.1.5 Discussion

The results from all studies combined suggested that, overall, remission was obtained in 59% of patients (95% CI: 44% to 73%).There was considerable heterogeneity between the different studies, with statistically significant heterogeneity and a high I^2 value of 88%.

The results for different types of medication showed varying results, ranging from a 0% remission for FMT and up to a 77% remission for steroids. For most, although not all types of medication, there was considerable heterogeneity between studies.

Antibiotics significantly induced remission in patients with chronic PIP with 70% remission rate (95% CI: 50% to 90%). Biologics significantly induced remission in patients with chronic PIP with a 53% remission rate (95% CI: 30% to 76%). Bismuth significantly induced remission in patients with chronic PIP in 41% (95% CI: 0% to 100%) but had a large confidence interval suggesting that the true effect is not known. Steroids induced remission in 77% of patients (95% CI 62% to 92%) but failed to achieve significance (p=0.75). Tacrolimus induced remission in 72% (95% CI 45% to

100%) but failed to achieve significance (p=0.57). Alicaforsen and elemental diets had remission rates of 58% (95% CI 28% to 85%) and 71% (95% CI 29% to 96%) respectively but these were based on a single study. FMT failed to achieve remission in patients with chronic PIP with remission rates of 0% (95% CI: 0% to 37%) in a single study.

The treatment of chronic PIP with the aim of achieving remission remains a challenge. This likely reflects our limited knowledge on the pathogenesis of PIP[206]. PIP not only causes morbidity to the patient but is also associated with financial and economic burden [207]. Antibiotics, usually in combination such as metronidazole and ciprofloxacin are generally first line therapy, with rifaximin and tinidazole being alternative agents to try in combination. Second line treatment options include corticosteroids such as beclomethasone or budesonide. Biologics including infliximab and adalimumab are third line agents that can be used to treat chronic PIP. Less well studied agents such as bismuth, tacrolimus and alicaforsen may be considered as alternatives to the above therapies.

Studies presented in this review must be interpreted with caution due to the small number of trials, lack of randomised placebo-controlled trials and small patient numbers. Only one of the studies was considered to be of moderate quality with the rest of the studies considered low or very low in quality. The lack of high-quality head to head trials makes it difficult to measure the benefit of one drug or agent over another and it is not possible to draw conclusions about the comparative efficacy of each agent. Due to small sample populations, it is also difficult to draw conclusions about the tolerability of each agent.

In many trials, there is a lack of agreement on what defines chronic PIP and what defines remission. A consensus definition of chronic PIP with standardised outcome measures would help the analysis and interpretation of the true efficacy of each treatment. Many studies only reported short term safety outcomes. It is also important to ensure that long term safety data is available for each agent and this should be taken into account when designing future studies.

A clinical algorithm based on the evidence in this review is suggested for the treatment of chronic PIP.

(See figure 6)

2.1.6 Conclusion

The treatment of chronic PIP remains difficult and is largely empirical. Our knowledge of the treatment of this condition is based on small studies with often poor study designs. Studies are mostly single centred with small patient numbers which reflects a condition that is rare and that requires specialist treatment. There is also a paucity of data exploring the long-term safety of some treatment options available to patients with chronic PIP. To improve data, larger randomised controlled trials will be beneficial. To overcome some of the limitations addressed in this review, a multi-centre, multi-national approach is needed.

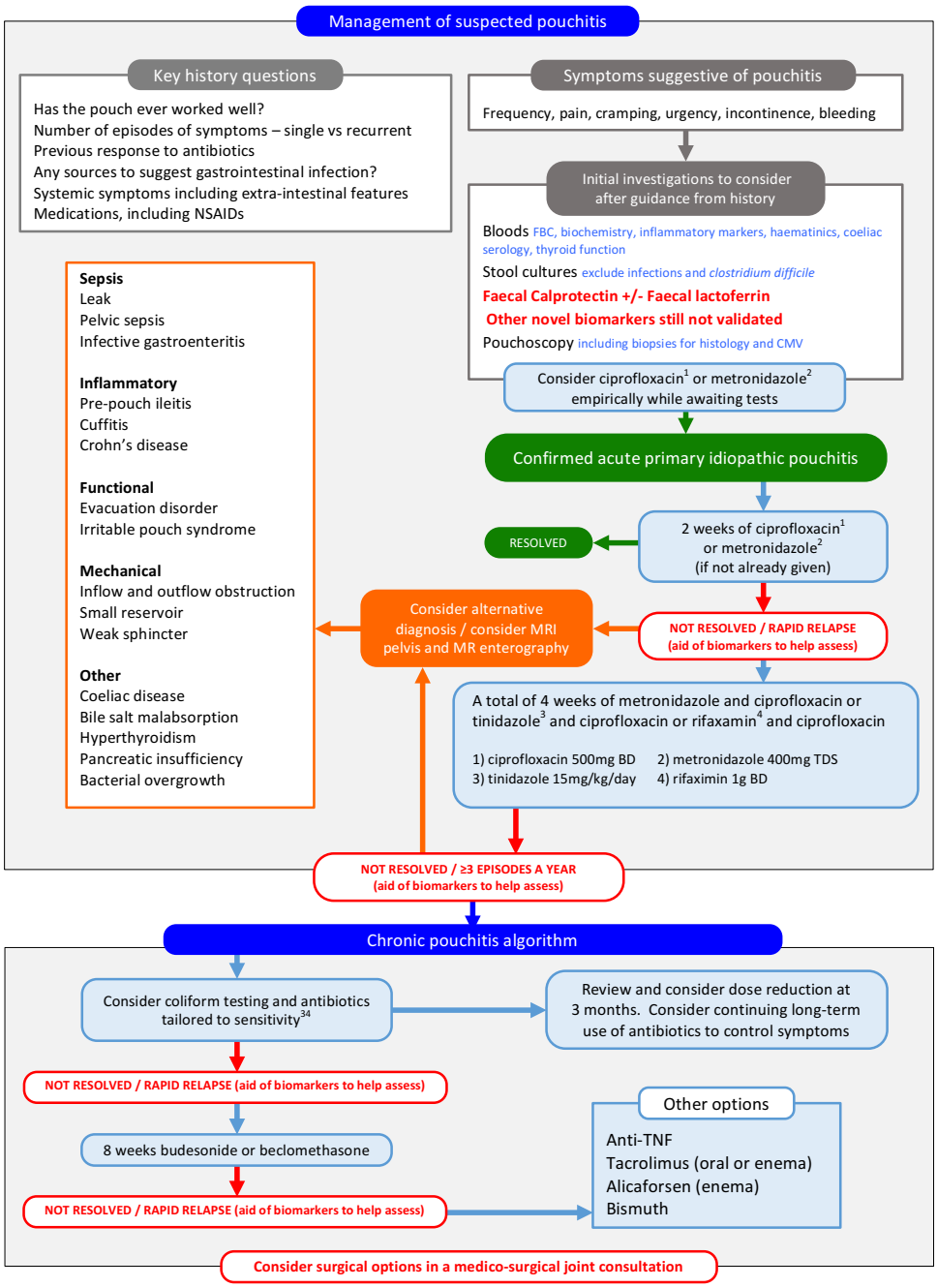

Figure 6. Management algorithm for chronic pouchitis.

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Chapter 3

Long-term antibiotics for primary idiopathic pouchitis

The above review highlighted that antibiotics are the mainstay treatment for both acute and chronic PIP. The treatment algorithm suggests that patients can be maintained on long-term antibiotics. However, the effect of this was not yet understood. The following section explores the long-term outcomes of long-term antibiotics for PIP.

3.1 Long term follow-up of the use of maintenance antibiotic therapy for chronic antibiotic dependent PIP

3.1.1 Introduction

As the last chapter discussed, chronic PIP develops in approximately 10–15% of patients with acute PIP and can be 'treatment responsive' or 'treatment refractory' to antibiotic therapy[209,210]. In practice, antibiotics are often needed to maintain symptomatic response and symptoms return upon withdrawal. This often results in patients being maintained on antibiotics long-term. Whilst antibiotics are the mainstay of treatment, there are currently no data on the efficacy and long-term safety implications of using long term antibiotics in chronic PIP. In particular, the use of ciprofloxacin has been associated with tendon rupture with an incidence of 2.4 per 10,000 patient prescriptions[211,212]. Metronidazole has been associated with peripheral neuropathy and although the incidence is unknown, total drug of greater than 42g or more than four weeks of treatment has been shown to be associated with peripheral neuropathy[213]. Furthermore, various antibiotic combinations have been implicated in *Clostridium difficile* diarrhoea. To the best of our knowledge, this is the first long-term study to explore these complications and effectiveness of long-term antibiotics for the treatment of chronic PIP.

3.1.2 Objectives

1. To assess the time from diagnosis of chronic PIP to pouch failure and in addition time to development of antibiotic resistance.

2. To assess adverse effects of antibiotics that occurred as well as the development of resistant organisms.

3.1.3 Methods

3.1.3.1 Study design

This was an observational study. I followed-up patients who were previously diagnosed with chronic antibiotic-dependent PIP. Data were collected from a single-centre which is a specialist centre for both pouch surgery and a tertiary centre for pouch referrals.

3.1.3.2 Participants

Patients were included if they met all of the following inclusion criteria:

- Aged ≥ 18 years
- Chronic antibiotic dependent PIP that had been maintained on antibiotics continuously for at least 1 year
- PDAI score >/= 7 at diagnosis
- Patients who had at least one clinic follow-up
- Patients were excluded if they met the following criteria:
- Patients with no follow-up data
- Patients with established Crohn's disease at start of antibiotic treatment
- A further 14 patients were identified during this study period and were also included.

3.1.3.3 Variables

Patients were followed-up until last clinic appointment or until pouch failure defined by the need for an ileostomy or pouch excision. Data collected included pouch failure, pouch-related complications, development of antibiotic resistance, development of C.*difficile*, tendon rupture, endoscopic and histological findings, and need for escalation.

3.1.3.4 Measurement of variables

Chronic antibiotic-dependent PIP was defined as the need for antibiotics for at least one month to control PIP symptoms (defined as a PDAI greater than 7) or the use of

antibiotics on three separate occasions to control PIP symptoms within a one-year period. Remission was defined as a PDAI of less than 7 on follow-up. Endoscopic scores were standardised using the PDAI. Stool coliform testing to identify antibiotic sensitivities and resistance was performed as described previously[214]. We assessed the time from diagnosis of chronic PIP to pouch failure and in addition time to development of antibiotic resistance. We also assessed adverse effects of antibiotics that occurred as well as the development of resistant organisms.

Medical records, endoscopic and histopathological reports were independently reviewed by two authors (JPS and Stephanie Poo (SP)). Baseline parameters collected included patient demographics, risk factors for PIP (a history of smoking, extra-intestinal manifestations, primary sclerosing cholangitis), symptoms (as reported by the patient) and mean stool frequency at baseline and at last follow-up.

Follow-up data recorded were: mean stool frequency at last follow-up, pouch failure as defined by need for an ileostomy, presence or absence of PIP, side effects of antibiotics, development of resistance to antibiotics and the development of *C.difficile*.

3.1.3.5 Study size

I retrospectively analysed 39 patients with chronic antibiotic dependent-PIP at a tertiary referral centre between January 2005 until April 2017. A further 14 patients were identified during this study period and were also included. Additional patients were identified through electronic interrogation of our endoscopy database, and patient notes using keywords to include “PIP”, “inflammation” and “chronic PIP”. Manual searches were also performed on surgical log-books and nurse led pouch clinic letters to highlight any additional patients.

3.1.3.6 Statistical methods

Continuous variables were compared using the student’s T-Test with categorical variables being compared using the chi-squared test. A binomial probability test was used to evaluate differences between our data and the reported incidence in the literature. Statistical significance was defined as p value <0.05. Statistical tests were performed using the STATA (StatCorp LP 4905 Lakeway Drive, College Station, Texas 77845-4512, USA)

3.1.4 Results

3.1.4.1 Participants and descriptive data

Patients' characteristics, presenting symptoms and associated risk factors are outlined in Table 3. The median follow-up was 102 months (range: 9-125).

Table 3. Patient characteristics, presenting symptoms and associated risk factors.

Variable	Total (%)
Total	**39** (100)
Male	**30** (77)
Female	**9** (23)
Median Age at presentation(years)	**42 (IQR)**(33-54)
Diagnoses	
Ulcerative colitis	**37** (95%)
Familial adenomatous polyposis	**2** (5%)
Presenting Symptoms	
Frequency	**26** (70)
Abdominal pain	**27** (73)
Per anal bleeding	**6** (16)
Urgency	**10** (27)
Bloating	**6** (16)
Incontinence	**9** (24)
Perianal discomfort	**7** (19)
Median 24h stool frequency (range)	**10** (4-25)
Risk factors	
Smoking	**0**
Primary sclerosing cholangitis	**1** (3)
Extra intestinal manifestations	**1** (3)

3.1.4.2 Remission vs failure rate

Of 39 patients, eight (21%) achieved symptomatic remission as defined by no longer requiring antibiotics while seven (18%) developed pouch failure, over a median duration of six years (range 3-9).

3.1.4.3 Antibiotics

Twenty-seven (69%) patients remained on antibiotics at last follow up; when excluding those who had pouch failure 27/32 patients (84%) who still had their pouch in continuity remained on antibiotics. The most common antibiotics used were ciprofloxacin (30%), metronidazole (19%) or a combination of both (11%) The other antibiotics included colistin, rifaximin and cefalexin. When considering pouch failure as last-follow up 31 (80%) patients remained on antibiotics continuously throughout the follow-up period.

A proportion of patients had tried other therapies to achieve clinical remission: biologics (n=3, 8%), steroids (n=10, 26%), probiotics (VSL#3) (n=3, 8%), elemental diet (n=13, 33%) and faecal transplantation (n=3, 8%).

3.1.4.4 Outcomes

Median stool frequency was unchanged from baseline at 10 per 24 hours.

3.1.4.5 Complications

Complications developed in a proportion of patients: fistula (n=7, 18%), perianal sepsis (n=6, 15%), and incontinence (n=5, 16%).

3.1.4.6 Hospital admissions

Thirty-six pouch-related hospital admissions occurred in 11/39 (28%) patients. Reasons for admission included: 23 admissions for obstructive symptoms in five patients, 11 elective examinations under anaesthetic in three patients, one developed incontinence and one developed an episode of bleeding from pouch.

3.1.4.7 Remission vs failure rate

Of 39 patients, eight (21%) achieved symptomatic remission as defined by no longer requiring antibiotics while seven (18%) developed pouch failure, over a median duration of six years (range 3-9).

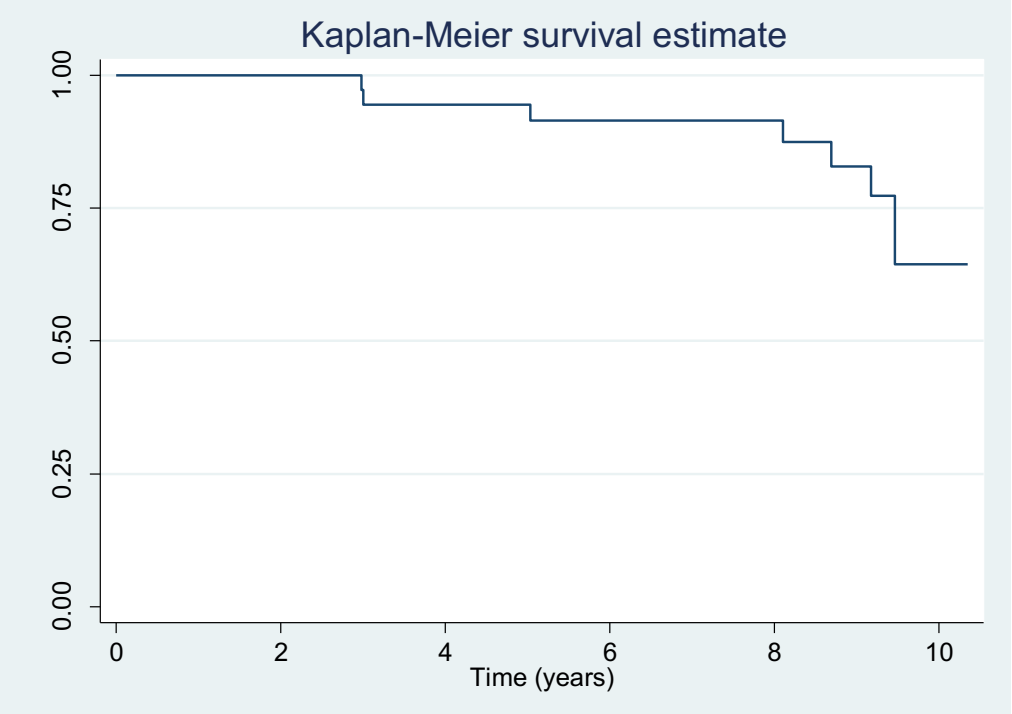

Figure 7. Kaplan-Meier survival estimate-pouch survival on long term-antibiotics.

3.1.4.8 Endoscopic examination

Mean number of pouchoscopies performed was seven (range: 0-19). Of all findings, the only significant change from initial to final pouchoscopy was the loss of vascular pattern (χ^2=7.5165, p=0.006).

3.1.4.9 Initial endoscopic findings

At initial endoscopy where antibiotics were initiated, 15 (39%) of patients had ulcers, eight (21%) of patients had a loss of vascular pattern, five (14%) of patients had pre-pouch ileitis, five (14%) had mucus exudate/oedema, two (4%) had erythema, two (4%) had friability and two (4%) had inflammation around the cuff.

3.1.4.10 Histological assessment

Twenty-eight (74%) had PIP identified from biopsy specimens taken at index pouchoscopy. Change in histological scores is tabulated in Table 4.

The change in scores was based on the Moskowitz scoring system[215]. This is based on an acute score which encompasses polymorph infiltration and presence of

ulceration and a chronic score which includes presence chronic cell infiltrates and degree of villous atrophy. Both the acute and chronic scores are graded as 0-6.

Table 4. Histological scores.

	First scope	Last scope	P value
Pathologist labelled PIP **(%)**	28/38 (74)	13/38 (34)	P<0.006
Mean total PIP score (range)	5.8 (1-10)	4.2 (1-10)	P<0.009
Mean total acute scores (range)	2.3 (0-6)	1.5 (0-5)	P<0.008
Mean total chronic scores (range)	3.4 (0-5)	2.6(0-5)	P<0.04

3.1.4.11 Antibiotic-related complications

Antibiotic-associated side effects occurred in 11 patients (28%): the majority developed intolerance or had worsening flares upon commencement of antibiotic, one developed dysgeusia and one developed peripheral neuropathy which resolved on withdrawal of metronidazole. No patient developed tendon rupture.

3.1.4.12 Antibiotic resistance patterns

Common organisms isolated from stool cultures included coliforms (n=24, 65%), *Escherichia coli* (n=18, 49%), Extended Spectrum Beta-Lactamase producing organisms (ESBLs) (n=12, 32%), and *Klebsiella* spp. (n=9, 24%).

Antibiotic resistance was identified from at least one stool sample in 28/36 (78%) patients. Common resistance patterns and the rate of desensitisation from available stool samples are detailed in Table 5.

Clostridium difficile toxin was not detected in any stool samples.

Table 5. Antibiotic resistance patterns.

Antibiotic	Resistance rate	Re-sensitisation rate
Ciprofloxacin	74%	32%
Co-amoxiclav	47%	25%
Trimethoprim	74%	12%

Antibiotic	Resistance rate	Re-sensitisation rate
Nitrofurantoin	15%	60%
Cefalexin	38%	23%

3.1.5 Discussion

My data showed that the long-term use of antibiotics achieve remission in only 21% of patients over a median follow-up of 102 (range: 9-125) months. Pouch failure in association with chronic PIP after a median follow-up of 8.5 years occurred in 18%. Side effects of long-term antibiotic use occurred in 28% of patients, with resistance to antibiotics from at least one stool sample occurring in 78% patients.

Remission rates in chronic PIP are variable with a meta-analysis highlighting that an overall remission rate following antibiotic use for chronic PIP was 70%[216]. When using a binomial probability test, I found that significantly less patients achieved remission ($P<0.001$). This suggests that the long-term use of antibiotics is poorly effective in achieving clinical remission for those patients with chronic PIP but may be useful at keeping symptoms under control. It may also suggest that chronic PIP is a separate entity and pathogenesis from acute PIP where treatment success with antibiotics is as high as 90%[216].

In my cohort, the only significant endoscopic change from initial pouchoscopy to last-follow-up was in loss of vascular pattern, which significantly improved when using antibiotics ($p<0.006$). This may represent the challenges in recording endoscopic findings whilst also suggesting that antibiotics may not improve endoscopic findings in chronic antibiotic dependent patients. Indeed previous data from our unit suggest that only 50% of patients achieve mucosal healing[217].

This study highlights that antibiotics significantly improved acute and chronic as well as total histological scores from first biopsies to most recent follow-up biopsies ($p<0.05$). This suggests that antibiotics may have a positive impact at the microscopic level in patients with chronic antibiotic dependent PIP. The impact of histological mucosal healing on pouch function long-term is unknown, but this data suggest that it is a poor surrogate marker as a measure of symptom free remission, as despite

histological healing only 16% of our patients achieved clinical remission in terms of avoidance of antibiotics.

The most common adverse effects associated with ciprofloxacin have been reported as gastrointestinal and neurological[218]. Ciprofloxacin has been reported as being associated with achilles tendonitis, tendon rupture. Metronidazole has been associated with peripheral neuropathies [212,219]. In this cohort, no patient developed achilles tendonitis. One patient developed peripheral neuropathy secondary to metronidazole use. Long term antibiotic use is associated with *C. difficile*[220]. In this cohort, despite long-term antibiotics, there were no episodes of *C.difficile*. Despite *C. difficile* being more widely recognized in the pouch[221–223], published data suggest that it is still a rare occurrence with one study reporting prior antibiotic use was not a strong predictor of developing *C.difficile*[221]..

In terms of patterns of antibiotic resistance, ciprofloxacin and trimethoprim had high rates of resistance with 25/34 (74%) patients developing resistance. Interestingly, resistance to antibiotics did not appear to be persistent with 8/25 (32%) patients in the ciprofloxacin group becoming re-sensitised to ciprofloxacin on subsequent stool samples without intervention.

3.1.5.1 Limitations

This study has a number of limitations. It is a retrospective analysis from a single institution with variability in endoscopic and histopathological reporting. My study is further limited by small patient numbers which may account for type 2 errors, as well as limited accounts of potential risk factors, symptomatology and change in antibiotics for each patient. Furthermore, patients developed other problems during the follow-up period which could account for some of the symptomatology.

3.1.6 Conclusion

The long-term use of antibiotics is poorly effective in achieving clinical remission where treatment is no longer needed to control symptoms. Long-term use of antibiotics for chronic PIP is associated with development of antibiotic related side effects and development of antibiotic resistance.

Although the use of antibiotics in chronic PIP may be justified, the utility of long-term antibiotics must be weighed against potential complications associated with PIP and antibiotics. Future work will benefit from prospective multi-institutional data collection, and the development of risk stratification tools for pouch survival in selected groups of patients.

Chapter 4

Biologics in Primary idiopathic pouchitis

My review highlighted that once antibiotics fail, biologics can be considered to salvage a pouch. The above chapter also suggests that long-term antibiotic use to manage symptoms is associated with potential problems and therefore other medications should be considered. The next chapter explores the outcomes of long-term biologics use for inflammatory pouch pathologies.

4.1 Long term outcomes of initial Infliximab therapy for inflammatory pouch pathology: a multi-centre retrospective study

4.1.1 Introduction

The rationale for the use of anti-TNF medications in chronic antibiotic refractory PIP and other fistulating conditions is that they have immunomodulatory qualities. The fact that PIP occurs more commonly in those who have a pouch for UC than for FAP suggests that both the immune system and its regulation have a role in the aetiology [24,224]. In the same way, pouch complications that mimic Crohn's disease (CD), such as deep ulceration, pre-pouch ileitis (PPI) or fistulation, are thought to be similar in aetiology and therefore, likely to respond [225].

Studies including small numbers of patients (often fewer than 20), and mostly retrospective in design, have demonstrated that infliximab (IFX) appears to have good clinical effectiveness in selected patients with inflammatory disorders related to the ileoanal pouch, achieving up to 80% short-term and around 50% long-term response[226]. However, few larger or prospective studies have been carried out to confirm this apparent benefit. Further investigation is therefore necessary to enhance our understanding of the benefit of anti-TNF therapy for both CD-like complications and chronic idiopathic refractory PIP. This study aimed to build upon the available literature by evaluating the effectiveness of IFX therapy for antibiotic refractory

idiopathic PIP and CD-like complications of the pouch and is the first to report long-term outcomes following IFX therapy beyond a year of follow-up.

Importantly, in the UK patients whom require a biologic will need funding from the clinical commissioning group which follow guidance from National Institute for Health and Care Excellence (NICE). Biologics are currently not NICE approved for pouchitis. To ensure these were funded an individualised funding requests form enabled the lead clinician to justify the use of a biologic in this patient group. These restrictions did not apply to Australia and funding was based on the clinicians' request.

4.1.2 Objectives

To assess the effectiveness of IFX for inflammatory disorders related to the pouch. The primary outcomes of interest were early IFX failure and secondary loss of response to IFX.

We separated the use of IFX into the following categories:

1. Antibiotic refractory idiopathic PIP with pre-pouch ileitis (PPI)
2. Antibiotic refractory idiopathic PIP without PPI

4.1.3 Methods

4.1.3.1 Study design

This was an observational, retrospective, multi-centre study.

4.1.3.2 Setting

In order to maximise the number of patients we included, data were collected between January 2016 and July 2017 from six centres in the United Kingdom and one centre in Melbourne, Australia. These included two tertiary referral centres and five district general hospitals. Patients were censored at the last clinical encounter following their most recent anti-TNF therapy or at point of pouch failure.

4.1.3.3 Participants

Patients were included if they met all of the following inclusion criteria:

- Patients who had undergone restorative proctocolectomy for UC

- Patients treated for an inflammatory disorder related to the pouch who were initiated on IFX medication and had failed at least four weeks of antibiotics prior to IFX therapy.
- Patients aged ≥ 18 years at time of initiation of IFX
- Patients who had at least one IFX infusion
- Patients who had one of the following reported outcomes within the last two months of their final IFX infusion in the study period

    - Pouch failure defined by need for an ileostomy
    - Need to switch to another anti-TNF agent or vedolizumab due to lack of efficacy
    - Need to stop IFX due to formation of antibodies or allergic reaction
    - Advised to continue on IFX therapy at last infusion

Patients were excluded if they met the following criteria:

- Patients with no follow-up data
- Patients with an ileoanal pouch for established CD

A template data collection sheet was given to each sub-investigator at each participating centre to collect the variables of interest. Potential patients were identified by using each hospital's anti-TNF and pouch databases. Those patients identified from the databases were then screened against the inclusion and exclusion criteria by interrogation of patient's electronic and case notes. Completed templates were then collated and analysed. We included only those patients who received IFX infusions to allow us to retrieve follow-up data. IFX was initially given at weeks 0-2 and 6 weeks at a dose of 5mg/kg, with 8-weekly administration thereafter. Dose adjustment was also included in the analysis.

4.1.3.4 Variables

Patients were followed up until their last clinical encounter or until the point of pouch failure. Censorship date was the 1st of July 2017.

4.1.3.5 Measurement of variables

IFX failure was defined as early IFX failure or secondary loss of response to IFX. Early IFX failure was defined as the need to switch to an alternative medication within eight

weeks of starting IFX due to lack of clinical efficacy, intolerable side effects or pouch failure within eight weeks of starting IFX. Secondary loss of response was defined as the need to stop IFX due to lack of clinical efficacy, development of antibodies to IFX, development of intolerable side effects or pouch failure beyond eight weeks of starting IFX. Pouch failure was defined by the need for an ileostomy or pouch excision.

Lack of clinical efficacy was agreed by a joint decision between the patient and senior clinician looking after the patient based on symptoms, endoscopic findings, histology and imaging.

Faecal calprotectin levels where available were recorded within six months prior to starting IFX therapy and at last available follow-up.

The use of the term CD is controversial in pouch-related inflammatory problems[52]. In my study we defined this by the presence of conclusive histology (granulomas supporting CD), or presence of skip lesions in the small bowel.

PIP was defined as being present in those patients who had a pouch disease activity index (PDAI) within one year before starting IFX of $\geq$7. PPI was defined as inflammation immediately proximal to the pouch inlet; inflammation was defined if the endoscopist reported the presence of oedema, ulceration, erythema or contact bleeding in the pre-pouch ileum on a pouchoscopy within a year before starting IFX.

Histological PIP activity score both prior to starting IFX (within one year) and at last endoscopic follow-up whilst on anti-TNF medications was measured (within one year). The change in score was based on the Moskowitz scoring system[215]. This index includes an acute score that encompasses polymorph infiltration and presence of ulceration and a chronic score that includes presence of chronic cell infiltrates and degree of villous atrophy. Both the acute and chronic scores are graded as 0-6.

4.1.3.6 Bias

Bias was limited by using objective measures to assess outcomes.

4.1.3.7 Study size

We retrospectively analysed data on 34 patients who fulfilled the inclusion criteria.

4.1.3.8 Statistical methods

Continuous variables were compared using the student's *t*-Test and the Wilcoxon-sign rank test as appropriate. Statistical significance was defined as a *p* value <0.05. Statistical tests were performed using the STATA (StataCorp LP 4905 Lakeway Drive, College Station, Texas 77845-4512, USA).

4.1.4 Results

There were 34 patients in my cohort. The median age of diagnosis of IBD was 25 years (range 5-53). The duration of IBD before pouch formation was 5 years (range 0-32). The median time from pouch formation to start of IFX was 125 months (range 0-308). The time from endoscopic examination confirming pouch inflammation to first IFX was 4 months (range 0-12 months).

Table 6. Baseline Characteristics.

Variable	Category	N (%)
Total patients		34 (100)
Gender	Male	19 (53)
	Female	15 (44)
Original IBD diagnosis prior to pouch formation	UC	30 (88)
	Indeterminate colitis	4 (22)
Extent of IBD prior to pouch	Pancolitis	33 (97)
Indication for pouch surgery	Unknown	1 (3)
	Failed medical therapy	33 (97)
	Toxic megacolon	1 (3)
Smokers whilst on IFX	Yes	2 (6)
	No	32 (94)
Primary sclerosing cholangitis	Yes	0
	No	34 (100)
Exposure to a biologic prior to pouch	Yes	1 (3)
	No	33 (97)

Table 7. Characteristics following pouch formation.

	Sub-category	Number (%)
Indication for IFX	Antibiotic refractory idiopathic PIP without PPI	18 (52)
	Antibiotic refractory idiopathic PIP with PPI	10 (29)
Basis of reclassification to “CD”	Reclassification to CD	6 (18)
	Imaging showing SB skip lesions	5 (13)
	Histology	1 (3)

All patients had trials of antibiotics prior to starting IFX. Seventeen patients had used budesonide and 10 had used azathioprine.

4.1.4.1 Treatment outcomes following IFX therapy

The median follow-up of the cohort was 311 days (range 1-130 months). There were a total of 18 IFX failures after a median 223 days, range (1-72 months).

Table 8. Infliximab failures.

Category	Sub-category	Number (%)
IFX failures	Total IFX failures	18 (52%)
	Early IFX failure	3 (8%)
	Secondary loss of response	15 (42%)
Failure by indication for IFX	Antibiotic refractory PIP without PPI	8/18 (44%)
	Antibiotic refractory PIP with PPI	4/10 (40%)
	Confirmed CD	4/6 (67%)
IFX failure within the first 365 days	Yes	13 (72%)
	No	5 (38%)

Ten patients who failed IFX underwent an ileostomy after a median follow-up of 258 days range (1-51 months). Twenty-four (71%) avoided a stoma after a median follow-up of 366 days (1-130 months). Of those that avoided a stoma, 15 (44%) continued on their IFX after a median follow-up of 260 days (1-129 months), four (12 %) were changed to adalimumab after a median follow-up of 413 days (5-18 months), three (8 %) were changed to vedolizumab after a median follow-up of 390 days (8-25 months), one achieved histological remission and managed to stop all treatment at 251 days and one was maintained on methotrexate and multiple antibiotics after 130 months.

The median time for those with early IFX failure was 42 days (range 1-42). All those with early IFX failure underwent an ileostomy without any further medications.

The median time for secondary loss of response was 280 days (range 3-47 months). Of those patients with secondary loss of response, four were switched to adalimumab and remained ileostomy free after a median follow-up of 413 days (range 5-18 months), seven required an ileostomy at a median follow-up of 204 days (range 3-47 months), four patients were switched to vedolizumab and were ileostomy free after a median follow-up of 14 months (range 3-37 months).

The cumulative ileostomy free survival from initiation of IFX at one, two, and four years was 82%, 76% and 70% respectively.

4.1.4.2 IFX failures by indication

4.1.4.2.1 *Antibiotic-refractory primary idiopathic pouchitis without pre-pouch ileitis*

Six of the 18 (33%) patients who had antibiotic refractory PIP without PPI underwent an ileostomy after a median follow-up of 148 days (42-280). One had early IFX failure to IFX and developed a rash at 42 days.

Seven of the 18 (38%) patients had secondary loss of response to IFX after a median follow-up of 204 days (82-385), 5/18 (27%) required an ileostomy after a median follow-up of 204 days (82-371).

Ten of the 18(56%) patients continued on their IFX and remained ileostomy free for a median of 78 days (42-464).

4.1.4.2.2 *Antibiotic-refractory primary idiopathic pouchitis with pre-pouch ileitis*

Three of the 10 (30%) patients who had antibiotic-refractory PIP with PPI underwent an ileostomy after a median follow-up of 35 months (14-47). One patient who had early IFX failure opted for an ileostomy after 42 days. Two patients had secondary loss of response due to loss of clinical improvement, after a median follow-up of 56 days (69-258), two of whom had an ileostomy after a median of 41 months (35-47). One patient went into histological and endoscopic remission after a follow-up of 251 days. Six (60%) patients continued on IFX and were ileostomy free after a median follow-up of 349 days (7-129 months).

4.1.4.2.3 *Confirmed Crohn's disease of the pouch*

The median length of time from pouch formation to reclassification to CD was 210 months (12-364). In those that were reclassified to CD, one had early IFX failure and was switched to Adalimumab and remained ileostomy free at one-month follow-up. Five of the six patients (83%) had secondary loss of response after a median follow-up of 290 days (154-498); three were switched to adalimumab and remained ileostomy free at a median follow-up of 328 days (154-498), two were changed to vedolizumab and remained ileostomy free at a median follow-up of 320 days (251-390).

4.1.4.2.4 *Fistulae of the pouch*

Controversy remains about anal and vaginal fistulae in patients with a pouch and what they represent[227]. It is possible that some of these are due to CD whereas others are caused by underlying pelvic sepsis, surgical complication or cryptoglandular sepsis distinct from CD[227].

In total three (9%) patients had fistulating disease, all of these also demonstrated small bowel skip-lesions or histological features of CD. Two patients had perianal fistulas and one pouch vaginal fistula. One patient who was maintained on IFX for nearly 11 years for UC with PPI demonstrated complete fistula healing of their pouch vaginal fistula on MRI at 125 months. One patient who had PIP without PPI showed no improvement in perianal fistula volume after 60 days of IFX but remained ileostomy free after a total of 113 days on IFX. One patient with PIP with PPI had no radiological follow-up but remained ileostomy free on IFX after nearly 11 years of follow-up.

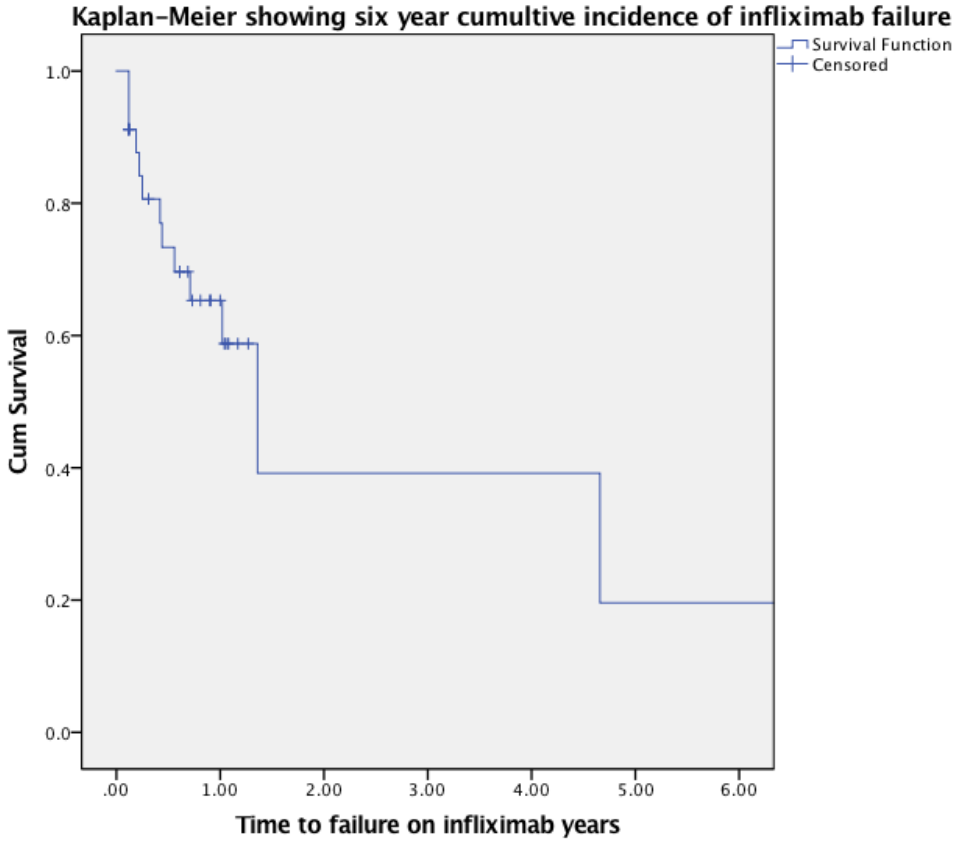

Figure 8. Kaplan-Meier plot of time to IFX failure.

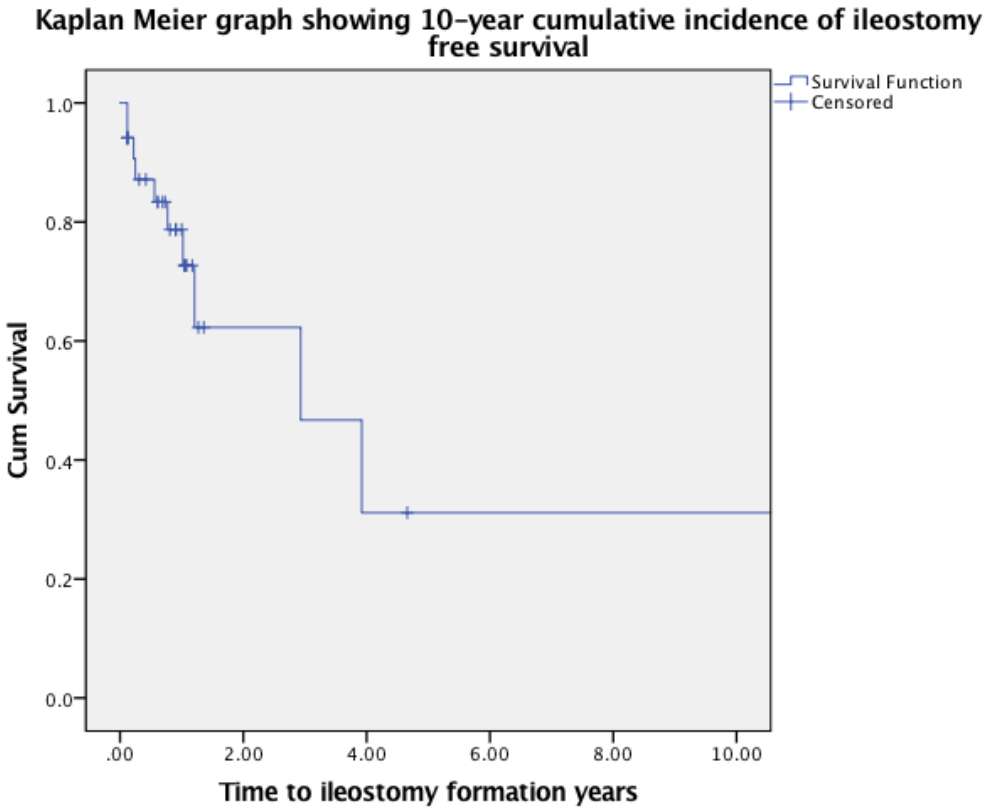

Figure 9. Kaplan-Meier plot showing 10-year ileostomy free survival.

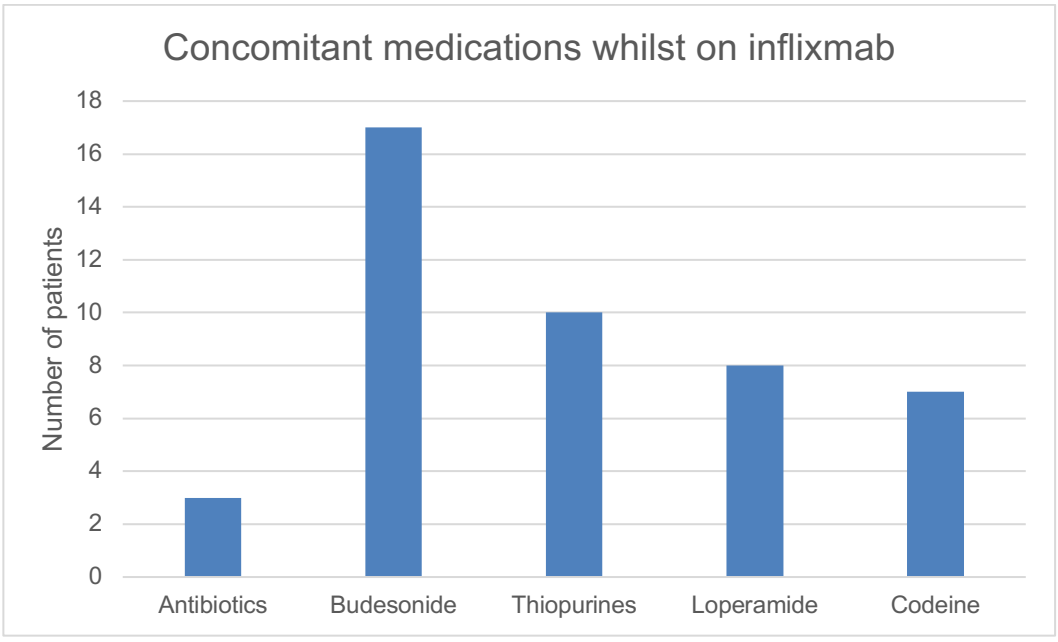

Figure 10. Medications used concomitantly to starting IFX.

Table 9. Effect of IFX on histology and faecal calprotectin.

	Prior to IFX initiation median (range)	After last dose of IFX	p-value
Acute histology score (n-13)	2.5	2	0.58
Chronic histology score (n-13)	4	3.5	1.0
Faecal calprotectin (n-13)	360	234	0.09
CRP	4.5	3	0.96

4.1.4.3 Safety of IFX medication

A total of eight hospital admissions occurred, of which six were considered to be associated with IFX use (three developed symptoms of sepsis, two developed obstructive symptoms and one developed shingles). Two patients were admitted for elective operations unrelated to their pouch symptoms.

4.1.5 Discussion

To my knowledge this is the largest study to explore the effectiveness of initial IFX therapy for inflammatory disorders related to the ileoanal pouch that extends beyond one year follow-up [152,153,228]. This is the first study to longitudinally follow-up the outcomes of these patients beyond a year.

My study has shown that initial IFX for inflammatory conditions in the ileoanal pouch is associated with an overall IFX failure rate of just over a half of all patients after a median follow-up of 223 days, range (1-72 months). In those in whom IFX failed, it did so in three-quarters within the first year, and over half of the patients required ileostomy formation to controls symptoms.

After initiating IFX therapy, twenty-four (71%) avoided a stoma after a median follow-up of follow-up of 366 days (1-130 months). It has been shown that the overall 5-year pouch survival is 95.6% (95% CI, 94.4–96.7)[72] and therefore my data suggest that once IFX is initiated for an inflammatory pouch problem, the chance of pouch survival is 24% lower over 5-years than the background population of patients with ileoanal pouch.

A systematic review on the use of biologics in PIP demonstrated that evidence from prospective, randomised-controlled studies of biologic use in this setting is currently unavailable[229]. A meta-analysis of observational studies found that anti-TNF medications were associated with clinical remission in 50% of patients, but this was based on small numbers[208]. Whilst it is difficult to report on clinical remission in my study due to study design, over two thirds of my cohort avoided a stoma after a median follow-up of 366 days (1-130 months) suggesting that the benefits of biologics can extend beyond ten years. Furthermore, two patients managed to come off biologics once symptoms had improved.

In terms of informing clinical practice, despite the advent of biosimilars, IFX remains an expensive treatment with important safety implications (as evidenced by the need for admission in six cases) including infection and cancers[230]. The high rate of treatment failure in my cohort suggests that it should be used only after careful consideration of alternative options and the potential complications. Furthermore, my study suggests that IFX resulted in no statistically significant changes in faecal calprotectin or histological improvement, albeit with relatively small patient numbers. It is important to note that therapeutic drug monitoring was not part of routine practice at the time that the data were collected and drug levels of IFX were not available to guide therapy.

My study is limited by small numbers, and the retrospective nature of the study means that information was sometimes missing. Further studies are needed, which prospectively evaluate patients commencing anti-TNF medications with carefully documented outcome measures as well as studies investigating newer biologics, such as vedolizumab and ustekinumab. It should be noted that early data, in the form of case reports and small case series, are now available suggesting a beneficial effect of vedolizumab in chronic antibiotic refractory PIP [231–234]. Larger studies may be able to highlight potential risk factors that predispose to treatment failure and therefore enable risk stratification. Another important aspect is to consider quality of life in future studies comparing those that continue on anti-TNF medications against those who opt for early alternatives such as ileostomy formation.

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4.1.6 Conclusion

Initial IFX therapy for pouch inflammatory conditions is associated with IFX failure of just over half of all patients. Despite a high failure rate, an ileostomy can be avoided in almost three quarters of patients at four years by switching to other medical therapies. However, patients should be carefully counselled about a high incidence of failure and therefore alternatives, including anti-integrin therapies and surgery, should be considered.

Chapter 5

Pre-pouch ileitis- is it just Primary idiopathic pouchitis or Crohn's disease?

A second major inflammatory pathology related to RPC is a condition called pre-pouch ileitis (PPI). From my chapters it became evident that often this was not considered as a separate entity from PIP. I therefore conducted a study in an attempt to understand this phenomenon separately and to understand treatments available and management. Importantly, much controversy still exists as to the underlying aetiology of this condition. As PPI affects the small bowel it has been suggested that PPI is pathognomonic of Crohn's disease (CD) and not UC[60,61]. In these studies CD has been diagnosed on the basis of ulcerated lesions of the small bowel or afferent limb[60] or if the colectomy specimen had either non-necrotizing granulomas or transmural lymphoid aggregates in areas that were not deeply ulcerated[61]. More recent reports, however have suggested that PPI is not associated with CD[62]. Moreover, our group reported that PPI has histological features that are consistent with UC[62]. Recently a large study has suggested that CD of the pouch is an overused diagnosis of exclusion and often this 'diagnosis' poorly correlated with histological findings[227].

Pre-pouch ileitis (PPI) has no standard definition but is characterised by the presence of mucosal inflammation of the ileum immediately proximal to the pouch. The estimated incidence of PPI is 6%[62,155]. This pattern of inflammation can extend for a significant distance into the afferent limb (up to 50 cm)[156] but this is unusual[62]. Much like PIP, it is a phenomenon that is almost exclusive to those patients that have RPC for UC and is less often seen in patients who have RPC for familial adenomatous polyposis (FAP)[156].

5.1 Methods

I followed up the original cohort of patients diagnosed with PPI that was described by McLaughlin *et al in* 2009[62]. Data originally collected from this cohort included

previous use of biologics, smoking status, stool frequency, endoscopic findings, treatment, symptoms and presence of terminal ileal (TI) inflammation prior to colectomy. Patients lost to follow-up were excluded, and the original data on the remaining patients were re-analysed.

Patients were censored at last clinic follow-up or at time of pouch failure. Pouch failure was defined as the need for permanent ileostomy. Follow-up data collected were stool frequency, endoscopic findings, treatment and overall pouch function.

PPI was defined as any endoscopically noted inflammation in the ileum proximal to the pouch. This included friable granular mucosa, mucous exudate, superficial ulcers, aphthous ulcers, and confluent superficial ulceration or based on histological presence of inflammation or inflammatory cells.

The aim of this study was to try and establish if this condition was different to PIP, what the long-term implications of this condition are and to see how strongly this condition was associated with Crohn's disease.

5.1.1 Statistical analysis

The primary outcome was time from pouch continuity to pouch failure. Kaplan-Meier was used to examine time from pouch continuity to pouch failure (Statacorp LLC Texas, USA).

5.2 Results

5.2.1 Patient demographics

The original cohort reported outcomes in 34 patients[62]. Three of these patients were lost to follow-up, leaving a total of 31 patients. Twenty-two (71%) were male. Median age at diagnosis of PPI was 42 years (range 35-54). Thirty patients had RPC for UC and one patient for FAP.

Table 10. Baseline characteristics of patients at diagnosis of PPI.

Variable	Category	Number (%)
Previous biologic use	No	30 (97%)
Smoker	Yes	1 (3%)
	No	30 (97%)

Variable	Category	Number (%)
TI inflammation noted prior to pouch surgery (+)	Yes	1 (3%)
	No	14 (70%)
Co-existing PIP with PPI	Yes	6 (30%)
	No	31 (100%)

(+) Values for 20 patients included

5.2.2 Follow-up

In the original cohort McLaughlin *et al* [62] reported a median follow-up of 12 months (range 2-47)[62]. The median length of follow-up from the index pouchoscopy of the 31 patients reported in this study was 98 months (range 27-143).

5.2.3 Last endoscopic follow-up

Twenty-eight (90%) patients had at least one follow-up pouchoscopy following the index pouchoscopy that diagnosed PPI. At last endoscopic follow-up, the median number of pouchoscopies was five (range 1-13). Seventeen (55%) patients had their PPI measured, the median length of PPI was 10cm (range 3-25). There were 16 patients who still had PPI, all of these also had co-existing PIP. PIP, diagnosed on both pouchoscopy and histology, persisted in 21 (68%) patients, suggesting evidence of chronic PIP. Whilst a direct comparison cannot be made it appears that PPI is associated with chronic inflammation in the pouch.

5.3 Symptoms at follow-up

Sixteen (52%) patients had chronic antibiotic dependent PIP, four (13%) had simple PIP, two (6%) had chronic antibiotic refractory PIP and nine (29%) patients were asymptomatic. Of the 24 patients whose pouch remained in continuity, the median stool frequency in a 24- hour period was nine (range 3-20).

In those treated with antibiotics, 12 (39%) patients reported worsening symptoms, 14 (45%) patients reported no symptomatic improvement and five (16%) patients reported symptomatic improvement since their index diagnosis of PPI.

5.3.1 Treatment at follow-up

Nine (29%) patients were receiving maintenance ciprofloxacin, two (6%) patients were maintained on ciprofloxacin and metronidazole, two (6%) patients were on ciprofloxacin and co-amoxiclav, one (3%) patient was on ciprofloxacin and rifaximin, eight (26%) patients were on no treatment, one (3%) patient was on nitrofurantoin and one (3%) was taking co-amoxiclav.

5.3.2 Reclassification

One patient subsequently developed a fistula-in-ano and one had multiple small bowel strictures. No patients had definitive histological evidence of CD, and these were the only two with features supporting reclassification as CD.

5.3.3 Pouch failure

Seven (23%) patients had pouch failure during the follow-up period. All of these required a permanent ileostomy; five (71%) were for chronic PIP and two (29%) were for small bowel obstruction due to pre-pouch stricture. Mean time from diagnosis of PPI to ileostomy in these patients was 81 months (SD 21.43). We compared rates of pouch failure with PPI against the incidence of overall pouch failure. We used the literature reported pouch failure rate of 10%[100,235,236] at 10 years (120 months). A binomial probability test showed that incidence of pouch failure is significantly higher in those patients with PPI ($p=0.03$) in my study with median follow-up of 98 months.

A graphical illustration of time to pouch failure is demonstrated in Figure 11.

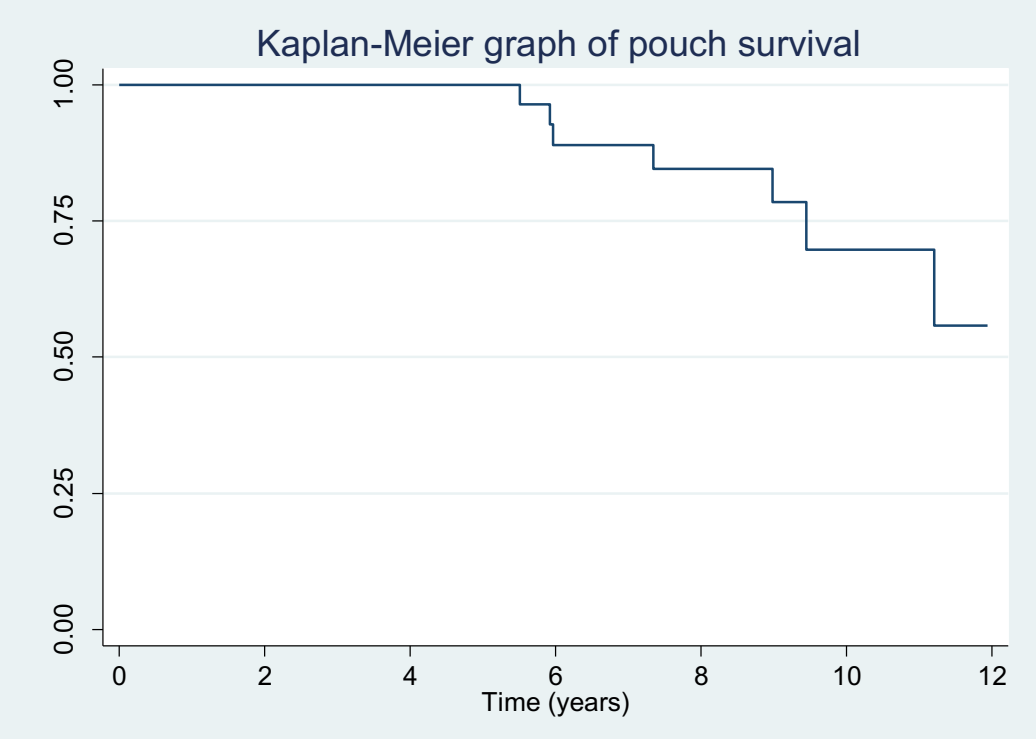

Figure 11. Kaplan-Meier of pouch survival in patients with PPI.

5.4 Discussion

My study demonstrated that in most cases PPI co-existed with PIP and therefore are likely to be part of the same aetiology. The current study suggests that PPI persists in 16 (58%) of cases with a median follow-up of 98 months (range 27-143).

In this cohort, of the 24 patients who retained their pouch in continuity, 22 (92%) required antibiotics at some point with 16 (73%) requiring chronic antibiotic use. This suggests that PPI may be associated with antibiotic resistant disease and a more severe form of PIP.

The overall cumulative incidence of pouch failure has been reported as 5% at 5 years and 8–15% after 10–20 years[100,235,236]. These data show that PPI is associated with a 23% rate of pouch failure at a median follow-up 98 months which is significantly ($p=0.03$) higher than pouch failure reported in the literature. During long-term follow-up, only two patients were considered to likely have CD. This supports my previous report that PPI is not strongly associated with CD[62].

This study has a number of limitations. It is a retrospective analysis of a prospectively maintained database. Due to the nature of the study there was some missing information which may have influenced the results. This study is further limited by small patient numbers.

5.5 Conclusion

PPI is associated with a significantly increased chance of pouch failure when compared with the overall reported literature for RPC ($p=0.03$). PPI does not appear to be a strong predictor of reclassification to CD. PPI occurs almost exclusively in patients with ulcerative colitis with co-existing PIP. Patients in this cohort with PPI had co-existing PIP, most requiring long-term antibiotics and remained dependent on antibiotics to control pouch symptoms.

Chapter 6

Biologics for pre-pouch ileitis

The previous chapter suggested that PPI is likely to be a continuum of PIP. It was also highlighted that long-term antibiotics are often necessary. If PPI was similar to PIP, it may be that biologic drugs are required when antibiotics no longer work. This chapter explores the role of biologics in pre-pouch ileitis.

6.1 Biological therapy for the treatment of pre-pouch ileitis: a retrospective experience from three centres

6.1.1 Introduction

It was shown in the previous chapter that PPI may share similar aetiology to PIP. As such it is possible that the immune system may play a role in PPI and therefore the use of anti-TNF medications is an attractive option that may help achieve remission. This follows the logic that if PPI is similar to PIP. Evidence has shown that biologic drugs can achieve remission in about 50% of patients with PIP[208].

The treatment of PPI as a specific entity has been poorly studied, but it is generally treated concurrently with PIP. One small study looked at the effects of antibiotics on PPI in a cohort with a diagnosis of UC and showed that 86% of patients showed symptomatic improvement with significant reduction in both stool frequency and length of PPI. However, this was based on only 14 patients. Furthermore, there has only been one small case series of five patients where Infliximab was found to be effective in the short-term for the specific treatment of PPI in a cohort with a diagnosis of Crohn's disease[237].

Historically, the limited literature reporting outcomes of biologic therapy for PPI has assumed that PPI is a Crohn's-like complication[152,237,238]. Recent studies, however, have highlighted that PPI is not a strong predictor for the development of unequivocal features of Crohn's disease [239,240] and therefore it is important to

report treatment outcomes dependent on whether Crohn's disease is thought to be the underlying cause or not.

This aim of this study was to document the effectiveness of biologics for the treatment of PPI. To my knowledge, this is the largest study to explore the effectiveness of all biologics for the specific treatment of PPI with the longest follow-up data.

6.1.2 Methods

This was a retrospective observational study across three centres. Data were collected from two centres in the United Kingdom and one centre in Bologna, Italy. This included two tertiary referral centres and one district general hospital. Patients were censored at the last clinical encounter following their most recent biologic therapy or until they had pouch failure defined by the need to form an ileostomy to relieve symptoms.

Biologics were funded in the UK through a standard biologics request form. As there was uncertainty about the aetiology of PPI we were able to achieve funding by considering this a disease similar to Crohn's disease. These restrictions did not apply to the Italian cohort and were funded on the clinicians' discretion.

6.1.2.1 Inclusion criteria

Patients were included if they met all of the following inclusion criteria:

- Undergone restorative proctocolectomy for ulcerative colitis
- Evidence of PPI on endoscopic assessment with inflammation confirmed histologically
- Treated with infliximab, adalimumab or vedolizumab

6.1.2.2 Outcomes

Patients were followed up until last clinical encounter. Outcomes included the presence of PPI following biologic therapy, pouch failure defined by the need for an ileostomy, remission of PPI defined by the absence of any pre-pouch inflammation on endoscopic assessment within a year of biologic therapy and the need to switch to an alternative biologic. Primary non-response was defined as failure of clinical

improvement at 12 weeks of biologic therapy. Secondary loss of response was defined as lack of clinical response to biologic therapy after 12 weeks of treatment.

Lack of clinical benefit was judged by the senior clinician looking after the patient and was guided by symptoms, endoscopic, histological and biochemical markers.

A template data collection sheet was given to each sub-investigator to collect the variables of interest. Potential patients were identified using each hospital anti-TNF and pouch database. Those patients identified from the database were then screened against the inclusion and exclusion criteria by interrogation of patient's electronic and case notes. Completed templates were then collated and analysed.

6.1.2.3 Measurement of variables

The use of the term CD is controversial in pouch-related inflammatory problems[52]. In my study, we defined this by the presence of conclusive histology (granulomas supporting CD) and/or presence of skip lesions in the small bowel. PIP was defined using the pouch disease activity index[241]. Patients were classified as having PIP if their PDAI within one year before starting Infliximab was ≥7. PPI was defined as any inflammation immediately proximal to the pouch; inflammation was defined if the endoscopist reported the presence of oedema, ulceration, erythema or contact bleeding in the immediate pre-pouch ileum with histological confirmation of inflammation in that section.

6.1.2.4 Statistical methods

All variables were analysed using STATA (StataCorp LP 4905 Lakeway Drive, College Station, Texas 77845-4512, USA).

6.1.2.5 Ethical approval

Ethical approval was granted by the West London NRES Committee IRAS ID: 23311 on the 4th September 2017. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the institution's human research committee.

6.1.3 Results

There were 29 patients in this cohort. The median age of diagnosis of UC was 27 years (range 6-48). The median age of the patients at censorship was 53 years, (range 19-68). The median time from pouch formation to diagnosis of PPI was 79 months (range 1-147). The median length of time a patient was on biologics at censorship was 12 months (range 2-62). The median length of follow-up on the whole cohort was 21 months (range 1-99).

Table 11. Baseline Characteristics.

Variable	Category	N (%)
Total patients		29 (100)
Gender	Male	17 (58)
	Female	12 (41)
IBD subtype at diagnosis	UC	28 (96)
	Indeterminate colitis	1 (4)
Smokers at time of censorship	Yes	9 (31)
	No	20 (69)
Primary sclerosing cholangitis	Yes	3 (10)
	No	26 (90)
Indication for biologic	PPI and PIP	22 (76)
	PPI in isolation	3 (10)
	CD with PPI	4 (14%)
Initial biologic used	infliximab	20 (69)
	adalimumab	9 (31)
Prior exposure to biologics before pouch	No	29 (100)

Ten patients were reclassified as having confirmed CD after a median time of 202 months following pouch formation, range 1-372. All these patients had their pouch originally for UC. Six had granulomas on further histological assessment and skip lesions on small bowel imaging and four had granulomas on histology alone.

6.1.3.1 Change of medication

One patient had primary non-response to infliximab and was changed to vedolizumab. Nine other patients had secondary loss of response to infliximab; of these six were changed to adalimumab and three were changed to vedolizumab. Of all those in whom the first biologic failed the median time to failure was 12.0 months (range 2-39).

6.1.3.2 Remission and pouch failure

At last endoscopic follow-up within one year of starting a biologic, 20/29 (69%) still had endoscopic evidence of PPI, seven (24%) had achieved remission and two had no endoscopic follow-up. Of the six (21%) patients who achieved endoscopic remission, four (14%) had a biopsy from the pre-pouch ileum which demonstrated histological remission. The other two (69%) were not biopsied at endoscopic follow-up. Of the seven (24%) that had achieved remission, five (17%) have stopped all medications and remained clinically well, one (3%) was taking colifoam enemas and on (3%) used cyclical metronidazole. All patients who had achieved remission were patients who had their biologic for PPI with co-existing PIP.

In my cohort 11(38%) patients went on to pouch failure after a median time from starting a biologic of 25 months (range 14-91). Of those who had their UC reclassified to CD, 3/10 (30%) had pouch failure compared with 8/19 (42%) who had UC (p=0.72) (figure 12). The cumulative 1, 2, 5 and 8-year failure rates were 0%, 17%, 30% and 38% respectively.

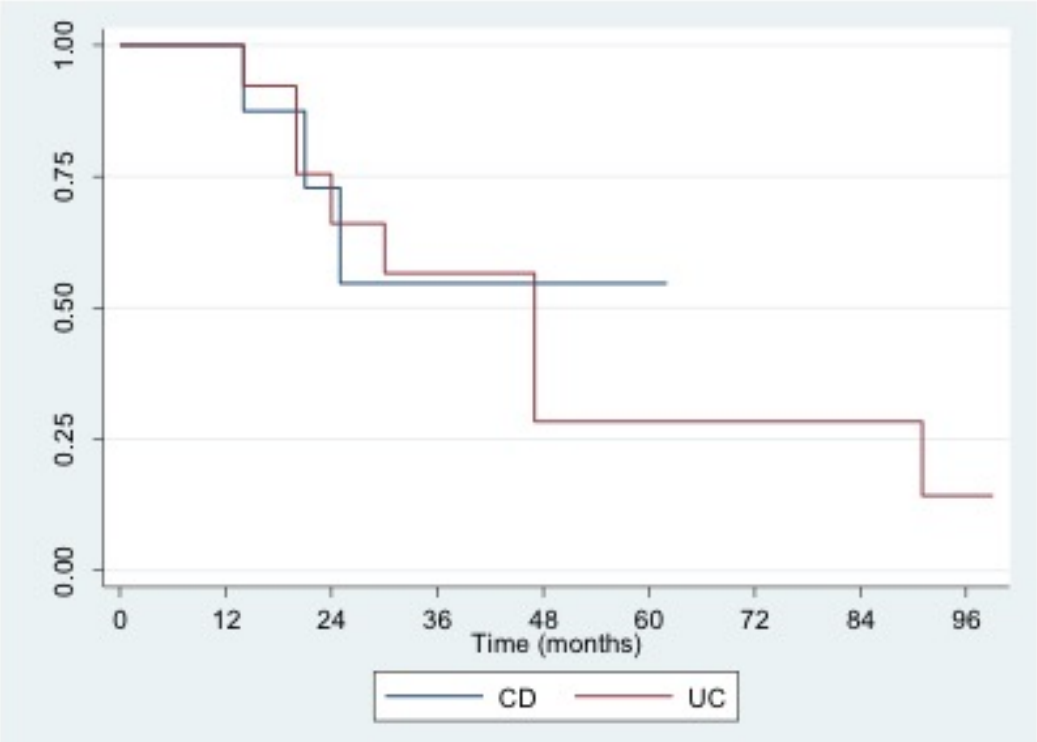

Figure 12. Kaplan-Meier graph for pouch failure:

Reproduced with permission from[242]

Log rank test comparing 'survival' times in the two groups p=0.72, no evidence of a difference between groups.

6.1.4 Discussion

Pre-pouch ileitis remains a difficult condition to treat. This study has highlighted that the use of biologics for PPI is associated with relatively low remission rates of 24% at a median follow-up of 20.5 months (range 1-99). When using biologics for PPI there was pouch failure in just over one third of the cohort after a median follow-up of 25 months (range 14-91).

There are a paucity of data highlighting the outcomes of biologic treatments in inflammatory pouch problems. A systematic review that included all chronic inflammatory pouch problems highlighted that remission could be achieved in 53%[216]. Just under a quarter of patients achieved remission from their PPI when using a biologic suggesting that the presence of PPI is associated with a less favourable treatment response.

It has been shown that PPI is often associated with PIP[62,239]. As such it is difficult to know what additional contribution to symptoms is made by PPI, or if PPI in isolation requires any different treatment from PIP, or indeed should be classified as CD, as it effectively represents a skip lesion. It has however been demonstrated that PPI is likely to be a poor prognostic sign and associated with higher rates of pouch failure[243].

This study has highlighted that PPI has practical and clinical implications. It is therefore essential that this complication is recorded during endoscopic pouch assessment.

This study is limited by small numbers and retrospective analysis. It is also limited by relative heterogeneity in patient cohort. Ideally, future studies should explore compare treatments for PPI in direct head to head trials. A standard definition of PPI would also aid my understanding and outcomes of this complication, which has been shown to have a poorer prognosis than PIP in isolation. Importantly, in the UK, funding biologics for PPI is currently unlicensed. Should PPI be considered a disease distinct from CD, it is likely that funding for this condition in the UK will become more difficult.

6.1.5 Conclusion

Biologics fail to achieve endoscopic remission of PPI in the majority of patients. In a small proportion of patients, they may help to prevent deterioration in pouch dysfunctional symptoms. In a large proportion of patients with PPI, surgery with indefinite diversion may be required despite biologic use.

Chapter 7

Incontinence and evacuatory problems in the ileoanal pouch

The previous chapters have highlighted that currently not all inflammation of the pouch can be successfully treated leading to significant problems to include incontinence and evacuatory problems. It was also evident that conventional medications such as antibiotics and biologics did not fully help solve some of these problems for patients with an inflammatory pouch. This led me to explore some non-medication-based techniques in improving symptoms related to a pouch behaving badly which focused on improving both incontinence and evacuatory disorders of the pouch.

7.1 Biofeedback in patients with ileoanal pouch dysfunction: a specialist centre experience

7.1.1 Introduction

Incontinence following can occur in up to 12%-31% of patients with an ileoanal pouch [244,245] but there are few studies reporting therapeutic interventions in this setting. Evacuatory difficulty in the absence of any mechanical or anatomical abnormality is uncommon and management options are limited.

Biofeedback therapy is a behavioural treatment which is non-invasive and offers a non-surgical approach as an alternative or adjunct for patients with functional bowel disorders[246]. The theoretical basis for biofeedback is ‘learning through reinforcement’ or ‘operant conditioning’[247]. Thus, biofeedback is classed as a re-education tool, in which information concerning a normally subconscious physiological function is relayed; the patient is then actively involved in learning to create a change in this function.

In a randomised controlled trial comparing treatment of faecal incontinence with either biofeedback or pelvic floor exercises in patients without a ileoanal pouch it was found that biofeedback was superior to pelvic floor exercises alone, with 76% of patients

having biofeedback compared with 41% who did not report adequate relief at three months ($p<0.001$) [248]. Furthermore, the treatment is widely accepted as a treatment for faecal incontinence[249]. Biofeedback has also been shown to be effective in evacuatory disorders[250], with one study highlighting that in 76% it can successfully treat obstructed defaecation[251].

This to my knowledge is the first study to report on outcomes of any intervention specific for ileoanal pouch related functional symptoms using validated questionnaires and builds on the available small case series that have used biofeedback for ileoanal pouch evacuation difficulties[252][253].

7.1.2 Methods

This was a retrospective single centre study. The notes of all 5027 patients who had attended biofeedback at St Mark's Hospital between January 2012 and October 2017 were reviewed to identify those who had been referred with an ileoanal pouch.

7.1.3 Variables collected

Patient demographics, details of ileoanal pouch construction and indication for biofeedback referral were recorded. ICIQ-B scores are routinely collected pre- and post- therapy in patients having biofeedback for faecal incontinence in my unit. Symptoms of incomplete evacuation are captured by my institution's evacuatory disorder questionnaire (appendix 1a) before and after biofeedback. Questionnaires were filled out independently by the patient both prior and after the biofeedback session.

The ICIQ-B is a validated questionnaire for faecal incontinence. It is split into four domains to separately measure bowel pattern, bowel control and quality of life with questions that are not directly scored but taken into account. It is a psychometrically robust, self-report instrument that evaluates anal incontinence and its impact on quality of life[254].

The tool used to measure evacuatory disorders was designed by my institution and is currently not published. It measures stool frequency and consistency of the stool according to the Bristol stool chart. The questionnaire also uses a binary scale to assess the feeling of incomplete emptying, the need to strain on evacuating and

presence of pain, bloating and laxative use. In both questionnaires, improvement is measured on an inverse linear scale.

7.1.4 Subjective outcome measures

Subjective markers of improvement were analysed in all patients who underwent biofeedback. These were identified by two independent reviewers (Heyson Chan (HC), JPS) by scrutiny of patient records at the next clinical encounter following their final biofeedback session. Subjective measures were grouped into “much improved”, “some improvement” and “no improvement” by each independent reviewer. Disagreements were resolved by consensus. We defined levels of improvement using real terms that the patient used.

‘No improvement’ was defined if the patient reported: ‘no improvement’, ‘still troubled’, ‘symptoms are the same’. ‘Some improvement’ was defined by the patient reporting: ‘some improvement’, ‘slight improvement’, ‘symptoms better’. ‘Much improved’ was defined by the patient reporting: ‘symptoms resolved’, ‘not bothering me anymore’, ‘improved a lot’, ‘symptoms much improved’, ‘good improvement’, ‘managing symptoms well’, ‘feeling much better’.

7.1.5 **Biofeedback technique**[254]

St Mark’s Hospital offers patients an individualised package of care to improve their bowel dysfunction, which includes any of the treatment options in the biofeedback therapy pathway (see below). Biofeedback takes a holistic approach, commencing with an advanced assessment to ascertain the patient’s symptoms and problems as well as their concerns and anxieties. This treatment therefore takes into account the physical and psychological needs of each individual patient, and as a result provides the therapist with an insight in to how symptoms can influence quality of life. A tailored-made biofeedback program is then formulated, taking into account the physical and psychological needs of each individual patient.

During the sessions, dietary and fluid modification are advised. Evacuatory techniques and posture are reviewed with the patient. Pelvic floor assessment with particular focus on pelvic floor exercise, urge resistance and myofascial release techniques are reviewed. These are practised with the patient several times during the session and

they are given printed materials to continue practising at home. In patients who needed psychological help, counselling sessions with a specialist are offered and coping techniques are taught.

Patients are usually offered up to six sessions over a period of 6-8 months, and are discharged when their symptoms resolved, they were satisfied with the outcomes, or they have made no progress in two or more sessions and further improvement deemed unlikely.

7.1.6 Statistical analysis

The Wilcoxon signed-rank test was used to analyse non-parametric pre-and post ICIQb. Statistical significance was defined as $p < 0.05$. The kappa co-efficient was used to assess the inter-rater agreement where subjective measures were assessed. Statistical tests were performed using the STATA (StataCorp LP 4905 Lakeway Drive, College Station, Texas 77845-4512, USA).

7.2 Results

Twenty-six patients who had ileoanal pouch related problems and underwent biofeedback were identified. Sixteen (62%) patients had predominately incontinence symptoms, eight (31%) had evacuatory disorder symptoms, one (4%) had abdominal pain and one (4%) had pruritus ani. There were nine (57%) patients who had objective follow-up data with a pre- and post-biofeedback objective questionnaire relating to faecal continence and five (63%) who had objective follow-up data with a pre-and post-biofeedback objective questionnaire related to evacuatory disorders and these are presented as a sub-analysis. The median number of biofeedback sessions was 2 (range 1-6). The median length of follow-up was 3 months (range 1-6). Baseline characteristics are shown in table 12.

Table 12. Baseline demographics of patients.

DEMOGRAPHIC		n (%)
Total patients		26 (100)
Age at start of biofeedback (median, range) (years)	49 (36-74)	
Male: female	Male	8 (31)
	Female	18 (69)
Reason for ileoanal pouch	Ulcerative colitis	23 (88)
	Familial adenomatous polyposis	3 (12)
Time from ileostomy closure to biofeedback (median, range) (years)	8 (1-33)	
Trial of dietary manipulation		18 (69%)
Trial of medical therapy before biofeedback	Laxatives	8 (31%)
	Anti-diarrheal agents	18 (69%)

Two patients had complications within 30 days following restoration of continuity, both had anastomotic dehiscence. Both culminated in incontinence.

Defecatory disorder using objective scoring system scores (n=4) (appendix 1a page 253-254)

7.2.1 Pre-biofeedback

The median stool frequency prior to biofeedback was 9 per 24 hours; the median consistency of the stool using the Bristol stool chart was 6.5; four (100%) patients complained of incomplete emptying; four (100%) reported straining; four reported pain (100%); three (75%) reported bloating; one (25%) required regular laxative use.

7.2.2 Post biofeedback

After biofeedback the median stool frequency was 7 per 24 hours, the median consistency was 5.5. Four (100%) patients complained of incomplete emptying, two (50%) patients reported straining, one (25%) patient reported pain, two (50%) reported bloating and laxatives were not required by any patient.

7.2.3 Incontinence disorder using ICIQB (table 13)

After biofeedback in those patients with a pre and post ICIQB score, biofeedback was associated with significantly improved quality of life (p=0.01) with improvements in bowel pattern and bowel control.

Table 13. Incontinence disorder using objective scoring system (n=5) ICIQB Scores.

Domain	Pre-biofeedback score (median, range)	Post biofeedback score (median, range)	P value
Bowel pattern	62 (49-62)	46 (39-62)	0.12
Bowel control	82 (33-102)	53 (11-76)	0.21
Non-scored	22 (17-35)	29 (12-29)	0.35
Quality of life	80 (62-98)	41 (30-55)	0.01

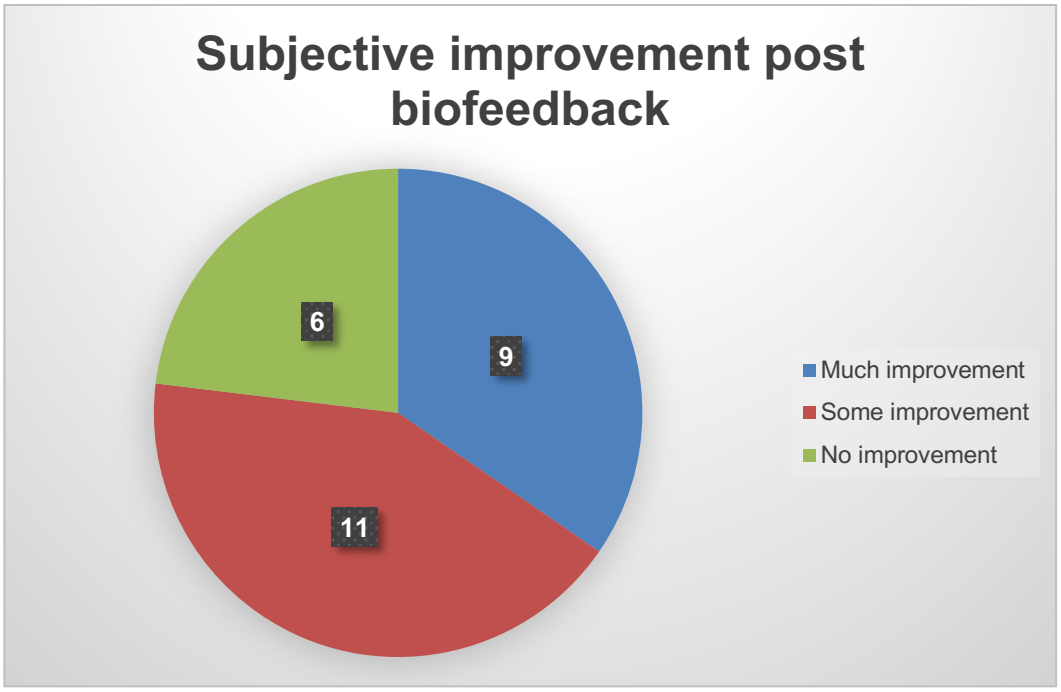

Figure 13. Overall subjective post biofeedback scoring.

The kappa-co-efficient for inter-rater agreement was 0.94 (SE 0.57) (reproduced with permission from[255]

7.2.4 Improvement by presenting symptoms (figure 13)

In the group with incontinence as the predominant symptom, four (25%) patients showed ‘much improvement’, eight (50%) showed ‘some improvement’ and four (25%) showed ‘no improvement’. In the evacuatory disorder group, four (50%) showed ‘much improvement’, two (25%) showed ‘some improvement’ and two (25%) showed ‘no improvement’. Biofeedback was associated with ‘much improvement’ in the patients with abdominal pain and pruritus ani as their predominant symptom.

7.3 Discussion

Biofeedback for predominately incontinence symptoms is associated with reduction in bowel frequency, an improvement in bowel control and statistically significant improvement in quality of life within six months of follow-up. Biofeedback shows potential in reducing pain, bloating and straining in patients with evacuatory disorders. Subjectively, biofeedback has at least some improvement in overall symptoms in 77% of all patients in my cohort.

There are sparse data on the value of biofeedback in the ileoanal pouch with one study showing that 9/11 patients had improvement in incontinence, defined as patients returning to a normal pouch defaecation pattern, following biofeedback[252]. Furthermore, a study that included the use of biofeedback for a variety of pouch conditions including PIP showed that in a subgroup of eight patients with non-relaxing pelvic floor dysfunction without PIP, all eight patients subjectively improved[253]. Therefore, my results are in keeping with these small studies that show biofeedback to be a useful adjunct to help these patients with functional problems related to an ileoanal pouch. Biofeedback has also been found to be effective in other forms of IBD with one study highlighting an 89% improvement in faecal incontinence in IBD patients following biofeedback[256] and another study showing a 30% improvement in constipation symptoms following biofeedback[257].

Patients suffering with symptoms of incontinence and evacuatory disorders are difficult to treat. This study has highlighted that biofeedback may be a valuable resource to help ileoanal pouch patients with incontinence, evacuatory disorders and functional problems. Furthermore, my study is the first to incorporate a validated questionnaire to assess outcomes in faecal incontinence in the pouch.

Limitations of this study were its retrospective nature. The study is also limited by small numbers, and the incomplete use of pre-and post-treatment measures. Nonetheless, the number of patients was comparable to other series available in the literature and builds on the limited data available. In addition, a significant improvement in quality of life was observed despite this sample size and therefore may suggest that in certain well selected patients' biofeedback represents a valuable treatment option but this will require further validation in prospective randomised studies.

Further prospective studies are required to evaluate biofeedback for incontinence, evacuatory disorders and other functional problems in patients with an ileoanal pouch. Furthermore, long-term appraisal of biofeedback for pouch related problems would help understand the long-term effects in this patient cohort. Randomised controlled trials comparing biofeedback against alternatives such as medications and surgeries such as sacral nerve stimulation may also provide insight into the effectiveness of biofeedback in the ileoanal pouch.

7.4 Conclusions

Biofeedback may be associated with significant improvements in quality of life for incontinence issues with the ileoanal pouch and may be associated with reductions in pain, bloating, straining and laxative use in patients with evacuatory disorders in ileoanal pouch. It is probably an underused service and may benefit more patients with similar problems. Further prospective studies are required to assess the efficacy in the ileoanal pouch.

Chapter 8

Efficacy and acceptability of a Renew® anal insert in patients who have undergone restorative proctocolectomy with ileal pouch anal anastomosis

The previous section highlighted that incontinence can cause a significant morbidity to a patient's life. Furthermore, there are limited treatment options. The next section explores a novel treatment for that management of incontinence for those that have undergone RPC.

8.1 Introduction

Treating anal incontinence in patients remains challenging. It has significant social and economic implications and can significantly affect a patient's quality of life[258–260]. It is likely that prevalence of faecal incontinence is underestimated due to patients reluctance to report it[261].

Incontinence following RPC has not been widely researched. In one study at 10 year-follow up, continence for stool and gas was present in 79.3% of patients, with 74.4% fully continent overnight. Incontinence following RPC can be multifactorial and be related to inflammation of the pouch (PIP), inflammation of the cuff (cuffitis), chronic sepsis, or anal sphincter weakness. Despite attempting to treat the underlying cause, incontinence may still remain a problem and symptomatic control may be necessary.

Treatment of faecal incontinence can include conservative approaches, such as lifestyle modifications and dietary manipulation, medications such as anti-diarrheal agents and barrier creams, and physical and psychological therapies such as exercise and biofeedback to surgery[262].

The Renew® anal insert (Renew Medical Inc, California, USA) is an inert single use silicon device which acts as an anal plug (figure 14). It is self-inserted using a removable applicator. The device is inserted into the anus where it acts as a seal. It is

then expelled during normal defaecation but can also be manually removed by pulling on the ring disc at the bottom of the applicator.

The device has been shown to be successful in 78% of patients with incontinence associated with a normal bowel, with 78% of patients being satisfied with the device[263]. This is the first study to assess the safety and efficacy of the Renew® anal insert in patients who have undergone RPC.

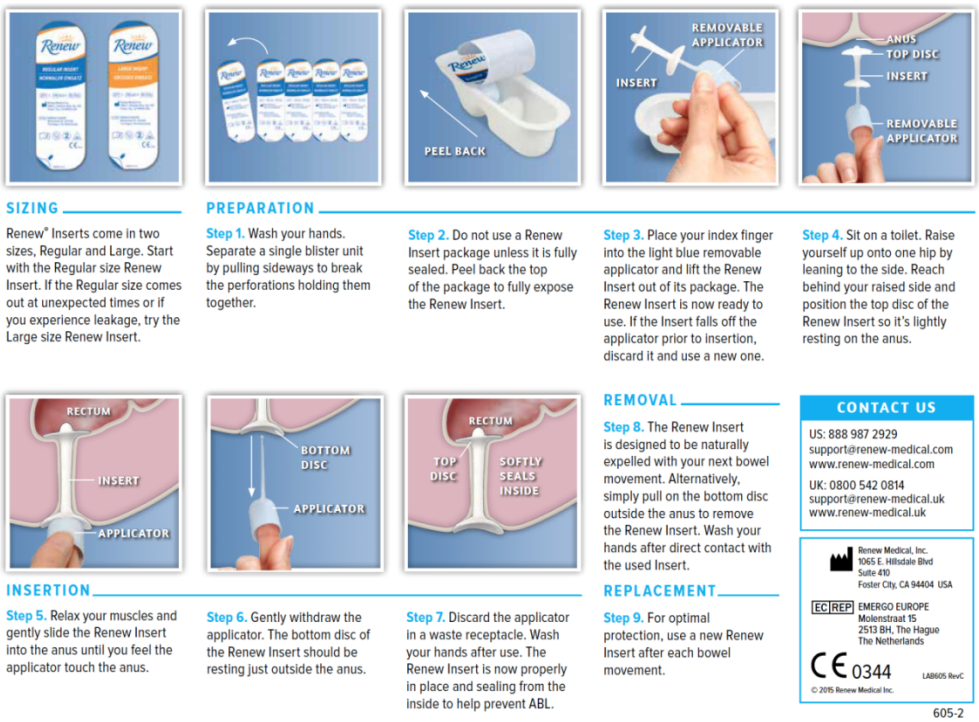

Figure 14. Renew® anal insert (reproduced with permission of Renew Medical Inc).

8.1.1 Methods

This was a prospective study exploring the acceptability and efficacy of the Renew® anal insert in controlling and improving incontinence in patients who had undergone RPC. This was a single centre study at a specialist centre.

Patients were identified through the biofeedback service at the hospital. Patients with ongoing incontinence issues where other causes have been excluded are referred into the biofeedback clinic. Patients were also identified from colorectal and inflammatory bowel clinics as well as through our local pouch nurses. Patients were included if they had undergone RPC for any reason and had self-reported passive incontinence for >2

weeks and were ≥18 years old at time of enrolment. Baseline clinical parameters were collected including age, reason for RPC, and other treatments given for passive faecal incontinence. Patients with active inflammation or undergoing treatment for pouch inflammation within 3 months of recruitment were excluded.

Patients with incontinence were asked to use the Renew® insert for 14 days following their standard biofeedback care. They were asked to keep a stool diary for 14 days and complete the standardised validated International Consultation on Incontinence Questionnaire-Bowels (ICIQ-B) questionnaire prior to commencing the trial of the Renew® insert and at the end of the 14 days.

Following completion of the study patients were asked to record their satisfaction and perceived efficacy of the Renew® anal insert device based on a three-point scale: satisfied, neither satisfied or dissatisfied, and dissatisfied.

The results were analysed on an intention to treat basis. If patients did not complete the trial or were lost to follow-up, we assumed that their ICIQB scores did not change from their baseline and that they were overall dissatisfied with the device and dissatisfied with the efficacy. Change in the ICIQB was calculated by subtracting average pre-treatment score from the post-treatment score.

8.1.1.1 Statistical analysis

The Wilcoxon signed rank test was used to compare the pre and post intervention scores. Statistical significance was defined as p-value <0.05. Statistical tests were performed using SPSS version 24, (International Business Machines Corporation, Armonk New York, USA).

8.1.1.2 Interventions

Patients who had self-reported incontinence were asked to use the Renew® anal inserts for a period of 14 days. The ICIQ-B is a validated questionnaire for faecal incontinence. It is split into four domains to separately measure bowel pattern, bowel control quality of life with questions that are not directly scored but taken into account. It is a psychometrically robust, self-report instrument that evaluates anal incontinence and its impact on quality of life[254].

All patients recruited were also invited to undergo endoanal ultrasound scan (EAUS) and ano physiology studies (AP) including anorectal manometry (AM) in order to obtain objective measurements of function.

Ethical approval was obtained from the London - West London & gene therapy advisory committee (GTAC) Research Ethics Committee (IRAS ID 211493).

8.1.2 Results

Fifteen patients were included in the study. There were 10 males and five females. The median age of the patients was 57 (range 24-74). All 15 patients had undergone RPC for ulcerative colitis. One patient was lost to follow-up.

A comparison of the pre and post-intervention scores was made, with the results summarised in table 14.

Table 14. ICIQB scores pre and post using Renew®.

Outcome	Pre intervention median, (range)	Post intervention median, (range)	P value
Bowel pattern	50 (25-70)	40 (31-70)	0.406
Bowel control	63 (29-82)	60 (10-82)	0.507
Quality of life	35 (10-66)	38 (18-66)	0.859
Other symptoms	31 (6-52)	32 (6-52)	0.953
Day seepage	1 (0-2)	1 (0-2)	0.581
Night seepage	1 (1-5)	1 (0-3)	0.034

The results suggested no statistically significant difference between pre and post intervention scores for the majority of the outcomes. The exception was for night seepage, where the values were significantly lower post-intervention compared to pre-intervention.

Eight patients were satisfied with the acceptability of the Renew® device, two were neither satisfied nor dissatisfied and four were dissatisfied with the results (figure 15). Six patients were satisfied with the efficacy of the device, two were neither satisfied or dissatisfied with the efficacy of the device and six were dissatisfied with the efficacy of the device (figure 16).

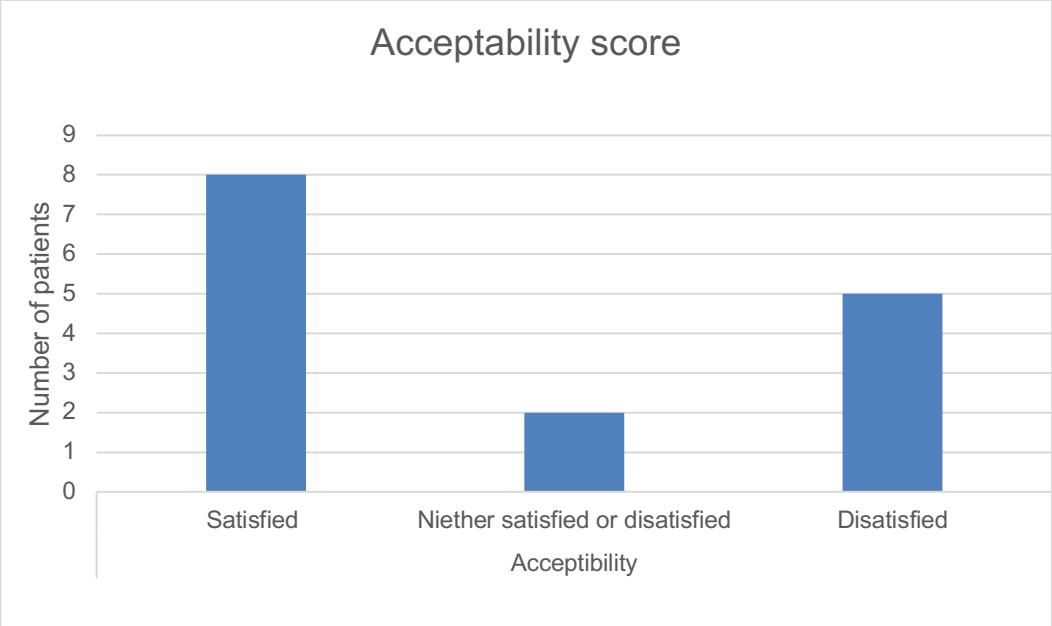

Figure 15. Acceptability of the Renew® anal device.

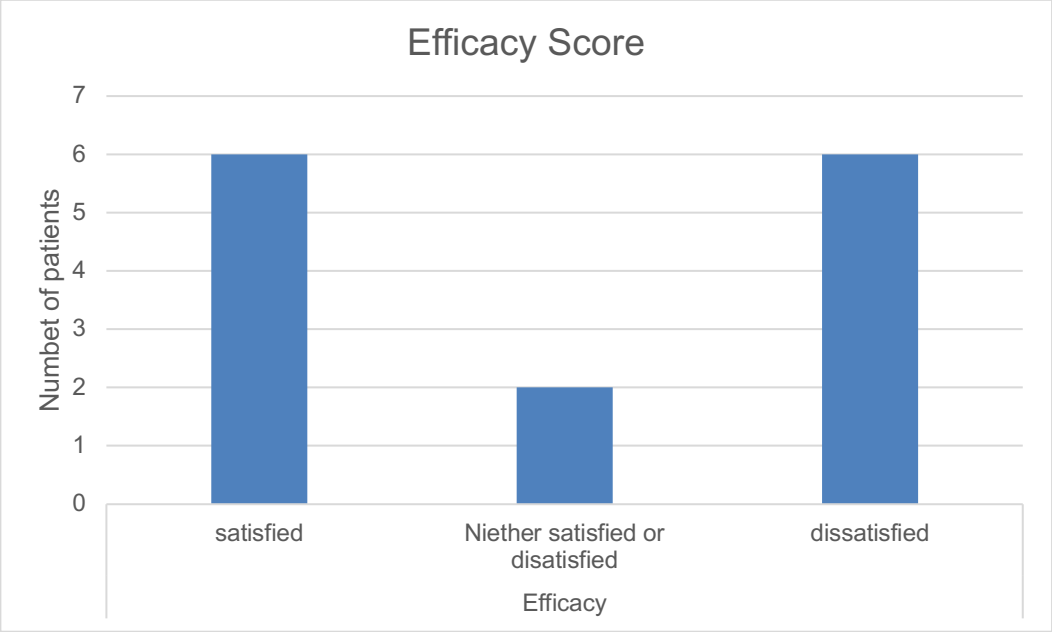

Figure 16. Efficacy of the Renew® anal device.

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8.1.2.1 Manometry results

Manometry was performed in ten patients. Four refused the test and one was lost to follow-up. Manometry showed low pressures in most of the patients. The median

resting pressure was 22.5 (20 - 73) mmHg. The median maximum squeeze increment was 97 (60 - 223) mmHg. The median endurance squeeze over 10 seconds was 53 (8 - 105) mmHg. The median for involuntary maximum squeeze was 96 (35 - 141) mmHg.

Endoanal ultrasound scan was performed in 8 patients. Seven patients did not attend clinic or they refused to have the test. Four patients had degeneration or defects in the internal anal sphincter contributing to their incontinence.

Table 15. Manometric values and EAUS results in patients dissatisfied with efficacy of Renew® anal insert.

IAS: internal sphincter. ES: external sphincter.

Patient number	Resting pressure	Max Squeeze	Mean Squeeze	Inv. Squeeze	Endurance	EAUS
1	23	114	91	36	8	No sphincter defects. Distorted and poorly defined IS
2	19	25	6	10	12	Poor definition of the IS with defect between 10 and 12 o'clock
3	21	60	39	51	35	ES and IS intact
4	45	159	114	132	78	DNA

Table 16. Manometric values and EAUS results in patients satisfied with efficacy of Renew® anal insert.

Patient no	Resting pressure	Max Squeeze	Mean Squeeze	Inv. Squeeze	Endurance	EAUS
1	73	184	111	141	105	ES and IS intact
2	22	189	167	60	55	
3	20	51	31	35	34	
4	20	51	31	35	34	
5	54	157	103	135	95	

8.1.3 Discussion

This study has shown that the Renew® anal insert can be a useful adjunct in the treatment of faecal incontinence in patients who have undergone RPC and is associated with a significant reduction in night seepage. The device was acceptable to 8/15 (53%) of patients and had efficacy in 6/15 (40%) of patients. Despite not reaching significance the anal Renew® anal insert was associated with a trend towards improvement in bowel control, bowel pattern and day time seepage.

Anal manometry tests confirmed low resting pressures in most of the cases. Some patients also had degeneration of the internal sphincter, which may have contributed to the symptoms. Interestingly in a small case series that followed up women with and without sphincter defects before and after RPC found that a sphincter defect was not predictive of long term incontinence[265]. This study suggests that both patients with intact and damaged sphincters suffer with incontinence following RPC and that patients with both an intact ES and IAS are more likely to respond well to the Renew® anal insert.

Normal pressures following RPC have yet to be established, however a study of 12 patients showed that patients with an ileoanal pouch had no significant differences in resting anal pressures when compared to healthy controls[266], suggesting that resting pressures are not altered following RPC. This is further supported by another study that suggested that there was no significant difference in anal manometry readings in patients with a colonic j-pouch and a coloplasty pouch[267]. However, this must be interpreted with caution in the absence of validated normal manometry readings in patients following RPC.

Previous studies have shown limited benefit in the use of anal plugs as they have been reported as poorly tolerated by patients and difficult to use[268][269]. It has been suggested that a major reason for this is the size of the plugs[263]. The Renew® comes in two different sizes and therefore may provide more comfort to patients with incontinence to standard anal plugs.

Faecal incontinence in patients with an ileoanal pouch is reported as much more common than in the general population; incontinence, it has been reported that 12 months following RPC 19% suffered with occasional daytime incontinence and 49%

suffered with nocturnal incontinence[101]. The reason for such a high rate of night-time incontinence is likely due to the sphincter muscles relaxing at night. My study has suggested that the Renew® anal insert can be particularly helpful with night-time incontinence.

The limitations of this study include the small sample size. As this was a pilot study a larger scaled study is needed, and power calculations could be based on the results documented here. Furthermore, one patient was lost to follow-up. We analysed the results as an intention to treat analysis which could have influenced the results.

Further larger studies should validate these findings and may be able to risk stratify those patients that may benefit from using the device based on baseline characteristics, physiology and other investigations. Future studies should help define normal manometry and endoanal ultrasound results in patients with a pouch, to help assess normal and diseased states.

8.1.4 Conclusion

This is the first study to show that the Renew® anal insert can be considered a treatment that can help patients who have undergone RPC with faecal incontinence. The Renew® anal insert can be both acceptable and efficacious and is associated with significant reduction in night-time seepage.

The previous chapters had identified that there are many inflammatory pouch related problems that for some are sub optimally treated. I therefore wanted to explore if I could understand some of the mechanisms that underpin the aetiology of inflammatory pouch related problems. I questioned if this approach may allow me to highlight potential novel therapeutic avenues as well as mechanisms behind the problems. To enable this, I used a combination of microbiota analysis and metabonomic profiling of patients who had undergone RPC. This will be explored in the next section.

Section 2:
Metataxonomic and metabonomic profiling of the ileoanal pouch

Chapter 9

Microbiota in the ileoanal pouch

9.1 Introduction

The resident gut microbiota is essential for a number of host physiological processes. These include digestion of dietary factors, development of the gut immune system and resistance to colonisation by pathogens[270]. The human gut microbiome is made up predominately of four major bacterial phyla: Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria[271,272]. Changes in, or imbalances of these are termed dysbiosis. Dysbiosis is linked to intestinal inflammation[273], with a decrease in bacterial diversity, or richness, being the most consistent finding in relation to disease activity [274–277]. Specifically, key changes have been identified in inflammatory bowel disease (IBD) such as a reduction in beneficial bacterial species including *Faecalibacterium prausnitzii*[278] and increases in more pathogenic species including members of Enterobacteriaceae[279,280]. Furthermore, studies have shown that in patients with IBD, the microbiota is different from healthy controls longitudinally and across all IBD subtypes[281]. Currently it is not understood if dysbiosis is the cause of, or the result of, intestinal inflammation.

IBD can develop at any age but tends to occur in the majority of patients within the first two decades of life[282,283]. It is difficult to assess changes in the microbiota prior to the development of IBD, as currently we are unable to predict those individuals who will develop the disease. To help understand the underlying aetiology of inflammation, it may therefore be beneficial to use a model to study the evolution of the microbiota over time.

The ileoanal pouch is a potential model to study pathogenesis of inflammation as PIP is common; moreover 40% of those that develop PIP do so within 12 months[284] of ileostomy closure. The relatively short time from ileoanal pouch formation to inflammation allows the convenient longitudinal study of the microbiota which gives insight into potential patterns occurring both in disease and non-diseased states.

Inflammation within the ileoanal pouch is seen less often in patients who have the same operation for familial adenomatous polyposis (FAP)[24,224], thus raising the possibility that PIP shares a similar pathogenesis to the inflammation that is seen in UC[285].

The role of the microbiota has been suggested to contribute to the pathogenesis of PIP[286], although its exact aetiology remains unclear. Novel laboratory techniques including next generation sequencing have transformed our understanding of the gut microbiota[287] although the exact bacterial taxonomical shifts in the ileoanal pouch have been difficult to interpret due to the heterogeneity in study designs, sampling techniques and analysis. This systematic review will explore the literature on the microbiota of the ileoanal pouch both longitudinally and in health and disease.

9.1.1 Advancements in technology

The majority of knowledge relating to the gut microbiota has been acquired through culture-based techniques, which are labour-intensive and not high-throughput. Furthermore, they require specific conditions to optimise bacterial growth (e.g. an anaerobic environment) which inevitably means that much of the gut microbiota is missed. The advancement in high-throughput comparative metagenomics enabled the development of next generation sequencing[288,289]. The invention and subsequent implementation of next-generation sequencing technologies have provided researchers with the apparatus and capabilities to analyse the gut microbiota without the need to culture microbes [290]. Several international studies and initiatives, including large-scale endeavours such as the Human Microbiome Project and MetaHit, have used these tools to identify over 1000 species within the gut, mainly belonging to four major phyla, namely: *Actinobacteria, Proteobacteria, Firmicutes,* and *Bacteroides* [291][292]. These large cohort studies also showed that relative abundance of these phyla differs between individuals [280]. However, the clinical and biological significance of these observations is currently poorly understood.

In the clinical context the gut microbiota interacts with the host immune system and metabolism[293–297]. It was highlighted that phylogenetic relationships, could be determined by comparing a stable part of the genetic code[298,299]. The 16s RNA gene sequence is considered the optimum gene sequence in phylogenetic studies as

the region is a highly conserved region which has a high tolerance to mutations and behaves like a molecular chronometer[299]. The 16s gene is also universal in all bacteria. The 16S rRNA gene sequence is about 1,550 base pairs long and is composed of both variable and conserved regions and is large enough with enough interspecific variability to undertake statistical analysis[300].

Figure 17. graphical representation of the 16s Rna gene with the v1-9 regions.

To try and understand the complexities of the changes in the microbiota in an ileoanal, a systematic review was undertaken.

9.2 Methods

9.2.1 Types of studies

Randomised controlled trials, cohort studies and observational studies were included. Studies which reported duplicate results were excluded. Those where data could not be extracted were also excluded.

9.2.2 Inclusion criteria

Studies were included if they reported microbiota analysis on either faecal samples or tissue from the ileoanal pouch and provided information on specific bacterial taxa.

9.2.3 Exclusion criteria

Studies were excluded if they did not report on patterns of individual bacterial taxa differences in the ileoanal pouch or if they reported on the microbiota of Crohn's disease or UC without any data on ileoanal pouch patients. We also excluded case reports and studies of fewer than ten patients.

9.2.4 Search methods for identification of studies

A search of the on-line bibliographic databases MEDLINE and EMBASE was carried out by two independent researchers (JPS and SO) to identify articles published between 1966 and February 2017. The following Medical Subject Heading (MeSH)

terms were used, which included both the root term and text words; ulcerative colitis, inflammatory bowel disease, IBD, idiopathic proctocolitis, gastrointestinal microbiome, bacteria, microbiota, microbiome, dysbiosis, bacteriotherapy, PIP, restorative proctocolectomy, ileitis. Synonyms and word variations were combined using the AND and OR function. Manual searches of the reference list from the potentially relevant studies were performed in order to identify additional studies that may have been missed using the computer-assisted search strategy. Abstracts from conferences of the American Gastroenterological Association, American Society of Colon and Rectal Surgeons, European Crohn's and Colitis Organisation, United European Gastroenterology and the British Society of Gastroenterology from 1965-2017 were also manually searched in order to identify unpublished studies.

9.2.5 Grading of studies

The GRADE system was used to assess quality of the studies[301]. Two independent reviewers (JPS and Sid Oke (SO)) assessed each study against GRADE standards and assigned a quality of evidence score of very low, low, moderate and high. Any disagreement was then solved by discussion and consensus.

9.3 Results

The search strategy found 844 references that were imported for screening. Five duplicates were removed. Eight hundred and thirty-nine studies were screened against title and abstract and 753 excluded. Eighty-six studies were assessed for full-text eligibility. Of these, 61 studies were excluded; 39 because of study design that did not fulfil the inclusion criteria (20 were review articles, 19 were abstracts containing less than 10 subjects), 20 did not report the required outcomes (10 did not report on individual taxa, three reported on bacterial metabolites, three reported on genetic changes, two described endoscopic outcomes, one reported immunological changes and one described tissue sampling techniques), one did not address the required patient population and one was not in English. After full screening, 25 studies were included. Manual reference searching identified a further paper, meaning there was a total of 26 papers included in the analysis.

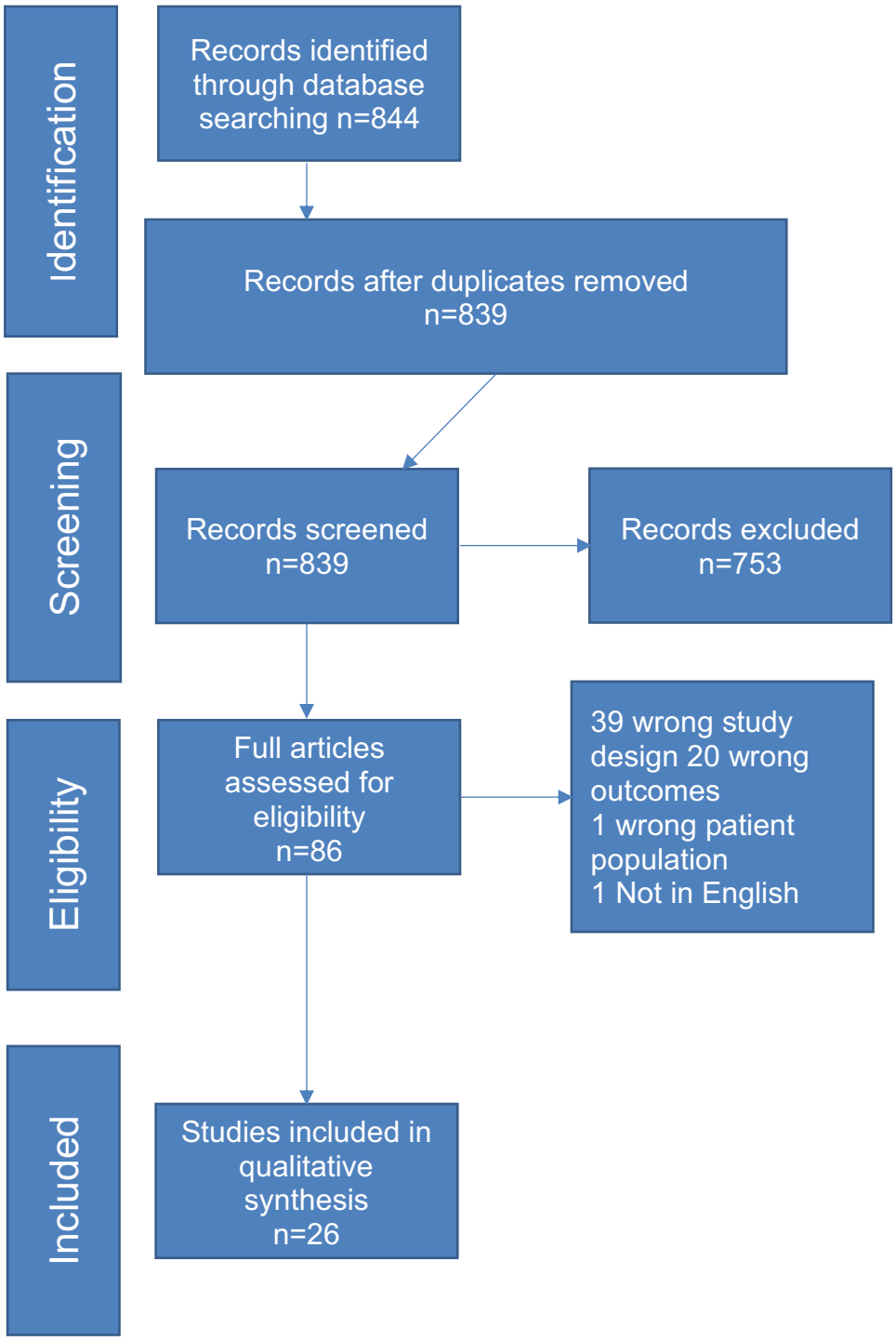


Figure 18. Prisma diagram.

9.3.1 Microbiota pre-pouch formation vs pouch

There were reported differences in the microbiota prior to undergoing the first stage of an ileoanal pouch operation (colectomy) between UC patients and FAP patients with lower bacterial diversity seen in mucosal samples in the colon prior to colectomy in

UC patients compared with FAP patients[302]. Smith *et al*[303] compared faeces from ileostomies in patients with UC and FAP prior to ileostomy closure and found there were significantly lower levels of *Clostridium perfringens* detected in the UC group[303] . As colonic bacteria are predominately anaerobic, it would be expected that anaerobic bacteria would be present in patients prior to colectomy. Various studies have supported this highlighting that anaerobes mostly predominate in faecal samples pre-colectomy [304]. Specifically, Almeida *et al*[305] found that *Veillonella* was the most prevalent bacterial species in mucus from the terminal ileum, colonic segments and rectum[305] from patients with UC. The authors suggest that the ongoing presence of *Veillonella* species in patients with UC may be associated with a persistently aberrant intestinal microbiota even in the presence of inflammation[305]. This study further characterised the differences in faecal microbiota across different colonic locations and found that both *Klebsiella and Lactobacillus* species were exclusive to a formed pouch and are not present pre-colectomy[305]. Further comparisons of faecal samples between a UC pouch and a UC ileostomy showed higher numbers of *Bacteroides* spp.*, Bifidobacteria* spp. [304] and *Enterococci* spp. [303] in the UC pouch.

9.3.2 Microbiota in the UC pouch: Non-inflamed or "healthy" vs inflamed

A non-inflamed or healthy UC ileoanal pouch has been characterised as having a higher bacterial diversity (richness) then an inflamed ileoanal pouch[306]. When biopsies were compared between healthy UC ileoanal pouches and PIP, *Bacteroides* spp.[307], *Clostridia* spp.[307,308], enterics such as *E. coli*, *Ruminoccoccus gnavus, Shigella* and *Streptococcus* spp.[308] were shown to be positively associated with a healthy ileoanal pouch. Another study, however, highlighted that *Clostridium* spp., *Eubacterium* spp., *Roseburia* spp.*, Escherichia* spp.*, Streptococcus* spp. and various sulphur-oxidising bacteria [309] were reduced in biopsies from a healthy ileoanal pouch.

9.3.3 Ileoanal pouch for ulcerative colitis vs Familial Adenomatous Polyposis

Smith *et al*[303] made comparison between faecal samples from ileoanal pouches formed for FAP and UC, it was found that anaerobic bacteria predominated in the UC ileoanal pouch[303]. Duffy *et al*[310] found significant decreases in aerobic bacteria in

faeces when comparing a UC ileoanal pouch with an FAP ileoanal pouch including *Lactobacilli* spp.; another study highlighted decreases in *Clostridium perfringens,* Bacteroidetes and *Bifidobacterium* spp.[310]. On a phyla level, ileoanal pouch biopsies showed Bacteroidetes belonging to Bacteroidaceae and Prevotellaceae [306] and Firmicutes belonging to Ruminococcaceae [306] were lower in UC. Biopsy samples showed higher levels of Proteobacteria belonging to Comamonadaceae, Moraxellaceae and Alcaligenaceae[306]. Faecal samples showed higher levels of Enterococcaceae and *Clostridia* spp

9.3.4 Microbiota in acute PIP

Similarly to IBD, bacterial diversity has been shown to be reduced in patients with PIP[206,305,311,312] with changes in both anaerobic and aerobic bacteria noted[313]. Analysis of faecal samples has shown higher levels of aerobes and lower levels of anaerobes in patients with PIP when compared with controls without PIP (both UC and FAP)[314].

Specifically, faecal samples[309,315–319] and biopsy samples[306,308,311,320] showed Bacteroidetes[309,311,315,320], Enterococcaceae[317,320], Lachnospiraceae[315,316], *Faecalibacterium* spp. [316], Ruminococcaceae ,*Streptococci* spp. [306,308], Alcaligenaceae[306] and *Bifidobacterium* spp. [315,318] were reduced in patients with PIP, whereas Enterobacteriaceae[306], *including E.coli* [306][319] Fusobacterium[308] and *Clostridia* spp. [309,318,319] were increased in patients with PIP[316]. One study highlighted specific increases in *Prevotella* spp*., Akkermansia* spp*.,* Firmicutes and Verrucomicrobia when comparing biopsy samples in acute PIP with FAP controls[309]. One study highlighted bacterial species that were exclusively found in PIP, including *Pseudoalteromonas* spp*., Desulfosporosinus* spp*., Microcystis* spp. *and Methylobacter* spp. [317]*.* It is important to highlight that *Microcystis* is a genus of Cyanobacteria, which is likely to have been introduced to the ileoanal pouch via the subject's diet and in bioformatic studies are often removed. Furthermore, *Methylobacter*, *Pseudoaltermonas* and *Desulfosporosinus* are not usual commensals of the ileoanal pouch and are found in soil and water[321] and therefore must be interpreted with caution.

9.3.5 Chronic PIP

Chronic PIP defined by the Heidelberg PIP activity score[322] or the need for long term medications to control symptoms[323,324] was associated with a significantly higher numbers of *Staphylococcus aureus* in faecal cultures and it has been suggested that this may be a responsible pathogen for chronic PIP[322]. Furthermore, faecal cultures have shown *Enterococcus* spp. [323], *Faecalibacterium prausnitzii*[324], *Clostridium* spp., *Ruminococcus* spp., *Eubacterium* spp., Lachnospiraceae and *Insertae Sedis XIV* are found in significantly lower numbers in chronic PIP patients[324]. Komanduri *et al,*[308] compared biopsies between an inflamed and non-inflamed pouch, they found reductions in both *Streptococcus* spp. and *Clostridium* spp. in chronic PIP[308].

9.3.6 Longitudinal changes in microbiota in the pouch

The microbiota of the ileoanal pouch has been shown to evolve over both the short-term and long-term in a manner that is unique to each individual[325]. Early microbiota changes have been demonstrated within two months of restoration of intestinal continuity, with colon-predominant anaerobic bacteria present in higher proportions in faecal samples, alongside a decrease in numbers of ileum-predominant species [326]. The most prevalent bacterial species found in faecal samples of the ileoanal pouch were Veillonella (90%), Enterobacter (70%), Klebsiella (70%), Staphylococcus (60%), Corynebacterium (60%), Peptococcus (60%), Clostridium (50%) and Lactobacillus (50%) [305]. Of these, Enterobacter spp. showed the highest mean concentration[305]. Almeida et al[305] compared faecal samples from the rectum pre-surgery and from patients with an ileoanal pouch two months post-surgery. They found Staphylococcus spp. and Corynebacterium spp. were found in less abundance (40% vs 70%) and (30% vs 60%) respectively, with increases in abundance of Bacteroidetes (30% vs 20%; p=0.049), Lactobacillus spp. (30% vs 0%; p=0.004) and Veillonella spp. (90% vs 30%; p=0.035) [305].

When the faecal microbiota of UC ileoanal pouch patients at two months after ileostomy closure was compared with the terminal ileum of healthy volunteer controls, *Enterobacter* spp. and *Klebsiella* spp., were significantly more prevalent whereas *Enterococcus* spp. and *Staphylococcus* spp. were more prevalent in controls[305]. There were decreases in the anaerobic bacteria *Clostridium coccoides* [305],

Clostridium leptum subgroups, the *Bacteroides fragilis* group, and also in *Atopobium* spp. [326] when comparing their prevalence in faecal samples prior to colectomy and after closure of the loop ileostomy. By six to 12-months after closure of the ileostomy, the prevalent species present in faecal samples from the ileoanal pouch were *E. coli*, *Veillonella* spp.*, Enterobacter* spp., *Klebsiella* spp. and *Peptococcus* spp. [305,322]. Of these, *E. coli* and *Enterobacter* spp. had the highest mean concentration[305]. The authors of this study concluded that the microbiota composition found prior to ileoanal pouch surgery for UC patients was similar to the composition found in the healthy ileoanal pouch after both two and eight months, following ileostomy closure. This suggests that the non-inflamed ileoanal pouch tended to recover to a microbial composition similar to pre-surgery values[305]. Studies looking at the faecal microbial diversity one year following ileostomy closure have shown that the microbiota composition had stabilized to reflect a more colonic profile[326,327]. This is supported by the decrease in *Lactobacillus* spp. and *Enterococcus* spp., which predominate in the small bowel microbiota [326].

9.3.7 Prevention and predictors of PIP

In analysis of faeces from patients with UC prior to ileoanal pouch formation, it has been seen that that a predominance of *Ruminococcus gnavus, Bacteroides vulgatus* and *Clostridium perfringens* and absence of *Blautia* spp. and *Roseburia* spp. can be predictive of PIP[328]. This was the first study to suggest that certain patterns in the microbiota can predict those who get PIP and those that do not. It will be important to repeat this study with larger numbers to potentially find patterns in the microbiota that predict those that may develop disease. This may help pre-operative counselling for a patient, whilst also giving a potential opportunity to alter the gut microbiota in order to prevent future complications with the ileoanal pouch.

9.3.8 Microbiota changes following treatment of PIP with antibiotics

The mainstay of PIP treatment is antibiotics. It is yet not fully understood the influence these have on disease course. Interestingly, Tannock *et al*[324] found that antibiotic administration (either ciprofloxacin, ceftin, cefuroxime or metronidazole) did not reduce the total number of bacteria in faecal ileoanal pouch samples[324]. In contrast, Kuhbacher *et al*[329], found that antibiotic use in PIP was associated with a lower

bacterial richness and diversity in biopsies from patients who achieved remission[329]. Furthermore, biopsies from PIP patients have shown that antibiotics reduce specific bacterial groups including Bacteroides, Firmicutes and *Tenericutes*[323]. Faecal samples from PIP patients have shown reductions in *Faecalibacterium* spp., *Roseburia* spp., *Coprococcus* spp. *and* Lachnospiraceae[316] Biopsies from PIP patients have shown increases in Enterococcaceae and Pasteurellaceae[316].

When comparing faecal samples from PIP patients who were using antibiotics with those not doing so, it was found that those not taking antibiotics had fewer Firmicutes and higher numbers of Proteobacteria[324]. In faecal samples from patients taking maintenance antibiotics for chronic PIP, Caulobacteriaceae, Sphingomonadaceae, Comamonadaceae, Peptostreptococcaceae, were significantly reduced[324].

With regard to the microbial impact of specific antibiotics, it has been shown that treatment with metronidazole resulted in complete eradication of anaerobic bacteria including *Clostridium perfringens*[319]. When PIP patients were treated with ciprofloxacin, *Clostridium perfringens* and all coliforms including haemolytic strains of *E. coli* disappeared[319]. Both ciprofloxacin and metronidazole are the first line treatments for PIP[208] and result in clinical remission in about 60% of patients[208]. As both *Clostridium perfringens* and *E. coli* have been associated with PIP[312,319] , this gives further credence to the concept that manipulating the gut microbiota to alter specific bacteria may help prevent this disease.

9.4 Discussion

Over time the pouch microbiota transforms into a more “colonic” typical phenotype after ileostomy closure. Similar to findings in IBD, a decrease in bacterial diversity and dysbiosis are associated with both acute and chronic inflammation. Changes in *Clostridium* spp. and *E. coli* have been shown to be associated with inflamed pouches, non-inflamed pouches and treatment response. Inconsistent findings across studies mean that it is difficult to assign a causative relationship of these changes with these phenomena.

There are many studies that highlight changes in bacterial composition in the ileoanal pouch, but these studies are limited by heterogeneity and in particular, analysis techniques and sampling strategies. On this basis, caution must be used when

interpreting microbiota data. Studies used a variety of methods to define microbial diversity. These methods can be broadly split into culture vs culture-independent approaches. Culture-based studies are likely to have a bias towards culturing more aerobically friendly microbes than exist in a true ileoanal pouch environment, thus over-representing aerobic bacteria whilst possibly under-representing anaerobic bacteria.

A significant limitation of studies in ileoanal pouch microbiota is accounting for confounders that influence the gut microbiota such as medications[330], diet[331], smoking and age. It has been suggested that PIP is not caused by a single factor[332] and the microbiota is just one of the factors that contribute to inflammation. This is obviously an issue with any disease and is something that cannot be avoided but should be considered when analysing the literature.

With advancing techniques in metagenomics, metaproteomics and metatranscriptomics, this may help to better understand the role of the microbiota in an ileoanal pouch both in health and disease.

9.5 Summary of the microbiota changes in the pouch adapted

9.5.1 Microbiota pre-pouch formation vs pouch

Colonic bacteria are found in the ileostomy and continue to predominate in the ileoanal pouch. Following ileostomy closure, anaerobic bacteria appear to be more prevalent in the ileoanal pouch.

9.5.2 Microbiota in the UC pouch: Non-inflamed or “healthy” vs inflamed

Bacterial diversity is important in maintaining a healthy ileoanal pouch with changes in Firmicutes and Bacteroidetes likely to play an important role in influencing a dysbiosis leading to disease.

9.5.3 Microbiota in UC vs FAP pouch

Consistent findings in an uninflamed UC ileoanal pouch compared with an un-inflamed FAP pouch include higher levels of Proteobacteria and lower levels of Bacteroidetes.

9.5.4 Microbiota in acute PIP

In acute PIP overall bacterial diversity is reduced. The consistent finding in acute PIP across studies is an increase in *Clostridium* species with more robust studies demonstrating a decrease in *Enterococcaceae.*

9.5.5 Microbiota in chronic PIP

Chronic PIP has been linked with an increase *Staphylococcus aureus*. In comparison with acute PIP, reductions in *Clostridium* species are a consistent finding in chronic PIP. As has been seen in ileal Crohn's disease, *Faecalibacterium prausnitzii* is reduced in chronic PIP.

9.5.6 Longitudinal changes in microbiota in the pouch

The ileoanal pouch microbiota transforms from a microbiota profile typically found in the small bowel to a more "colonic" microbiota over-time with both Enterococcus spp. and *Lactobacillus* spp. reducing in numbers over-time. These changes can occur as early as two months, with more stability in the microbiota noted with increasing age of the ileoanal pouch.

9.5.7 Microbiota changes following treatment of PIP with antibiotics

Antibiotic treatment for PIP is associated with an overall reduction in bacterial richness within the ileoanal pouch. Reduction in both *E. coli* and *Clostridia* species appears to be important in treating PIP but this requires further clarification.

Chapter 10

16s rRNA analysis of the ileoanal pouch

Following the systematic review, I wanted to analyse the microbiota in patients with chronic PIP to assess if there are patterns in the microbiota that may be associated with treatment response. To my knowledge this would be one of the first studies to explore the microbiota in the chronic PIP using metataxonomic techniques. The next section describes these experiments.

10.1 Methods

10.1.1 Clinical data

The following clinical data were collected from patients whom had undergone restorative proctocolectomy for ulcerative colitis : age, gender, past medical history, drug history including antibiotic use, age of pouch.

Patients with pouch dysfunction symptoms were seen in a specialised pouch clinic. Patients would undergo a series of tests to confirm chronic PIP defined by a pouch disease activity score ≥ 7. Symptoms of pouch dysfunction were required to be present for four weeks

Two groups of patients were assessed. Patients with chronic PIP already on antibiotics and those patients with chronic PIP not yet on antibiotic treatment. Patients off antibiotics were offered antibiotics and followed up 4 weeks following antibiotic use. Those patients on antibiotics already were offered the opportunity to come off antibiotics and again were followed-up at 4 weeks.

Those patients that came off antibiotics that flared were given the opportunity to restart the antibiotics to prevent deterioration in symptoms. For analysis purposes patients were analysed as either on antibiotics if they received antibiotics 2 weeks prior to the clinic or off antibiotics if they had stopped all antibiotics 2 weeks prior to follow-up.

10.1.1.1 Outcome measures

PIP was defined using the pouch disease activity index (PDAI)[241] and PIP was considered when the score was ≥ 7. Response was defined as either a 2-point reduction in a patients PDAI or a score of <7.

Patients were classified as off antibiotics if they had stopped all antibiotics for a period of at least 2 weeks prior to sample collection.

10.1.1.2 Stool

Stool samples were obtained at the time of consent. These were then titrated using sterile pipettes into Eppendorf tubes and stored at -80°c within 30 minutes of delivery of sample until further analysis.

10.1.1.3 Faecal microbiome analysis DNA extraction and 16S rRNA gene sequencing

DNA was extracted from stool using the PowerLyzer PowerSoil DNA Isolation Kit (Mo Bio, Carlsbad, CA, USA) following manufacturer's instructions, with the modification that samples were bead beat in for 3 min at speed 8 in a Bullet Blender Storm (Chembio Ltd, St. Albans, UK). DNA was extracted from 250 mg of stool and DNA was stored at -80°C until ready to use (a detailed technique can be found in supplementary materials).

Sample libraries were prepared following Illumina's 16S Metagenomic Sequencing Library Preparation Protocol[333] with the following modifications. First, we amplified the V1-V2 regions of the 16S rRNA gene using the following primers; 16S Amplicon PCR Forward Primer = 5'
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
16S Amplicon PCR Reverse Primer = 5'
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAAT
CC . The index PCR reactions were cleaned up and normalised using the SequalPrep Normalization Plate Kit (Life Technologies, Paisley, UK).

Sample libraries were quantified using the NEBNext Library Quant Kit for Illumina (New England Biolabs, Hitchin, UK). Sequencing was performed on an Illumina Misses platform (Illumina Inc., Saffron Walden, UK) using the MiSeq Reagent Kit v3 (Illumina) and paired-end 300bp chemistry. The sequences were loaded onto the

QIIME pipeline using the silva database. Statistical analysis was performed using STAMP 2.1.3 software with Welch's two-sided t-test for comparing two groups with false discovery rate correction. Microbial richness and diversity were calculated based on Chao1 index. Weighted Unifrac metrics were applied to construct PCoA plots.

10.1.1.4 Miseq processing

The Miseq files were then processed in Mothur version 1.35.1[334]. The 16s rRNA sequencing data generated on MiSeq was processed on Mothur using the MiSeq SOP Pipeline.[335] Sequence alignment was conducted using the Silva database (*www.arb-silva.de*) and sequences classified by the Wang method using the RDP reference database[336] Extended error bar plots are different taxanomic levels were generated in the Statistical Analysis of Metagenomic Profiles (STAMP) software package using White's non-parametric t-test with Benjamini-Hochberg FDR correction for multiple comparisons. Corrected p values <0.05 with effect size >1% were considered significant. The UniFrac weighted distance matrix generated on Mothur was analysed using the Vegan library within the R statistical package to produce non-metric multidimensional scaling (NMDS) plots and PERMANOVA p-values.The α diversity (Shannon Index) and richness (total number of bacteria observed) were calculated in Mothur and compared between groups using IBM SPSS Statistics Software version 23.

10.1.2 Hypothesis

1. There will be significant differences in microbiota between responders and non-responders
2. There will be significant differences between those patients on and off antibiotics

10.1.3 Results

There were 28 patients in the cohort , which compromised 23 patients with PIP and five healthy controls. There were 10 females and 18 males. The median age of the cohort was 47 years (range 26-74). The median age of the patients with PIP was 47 years (range 21-74) There were a total of 12 samples taken on antibiotics and 11 off antibiotics. In total there were 6 patients who had paired pre- and post -antibiotic

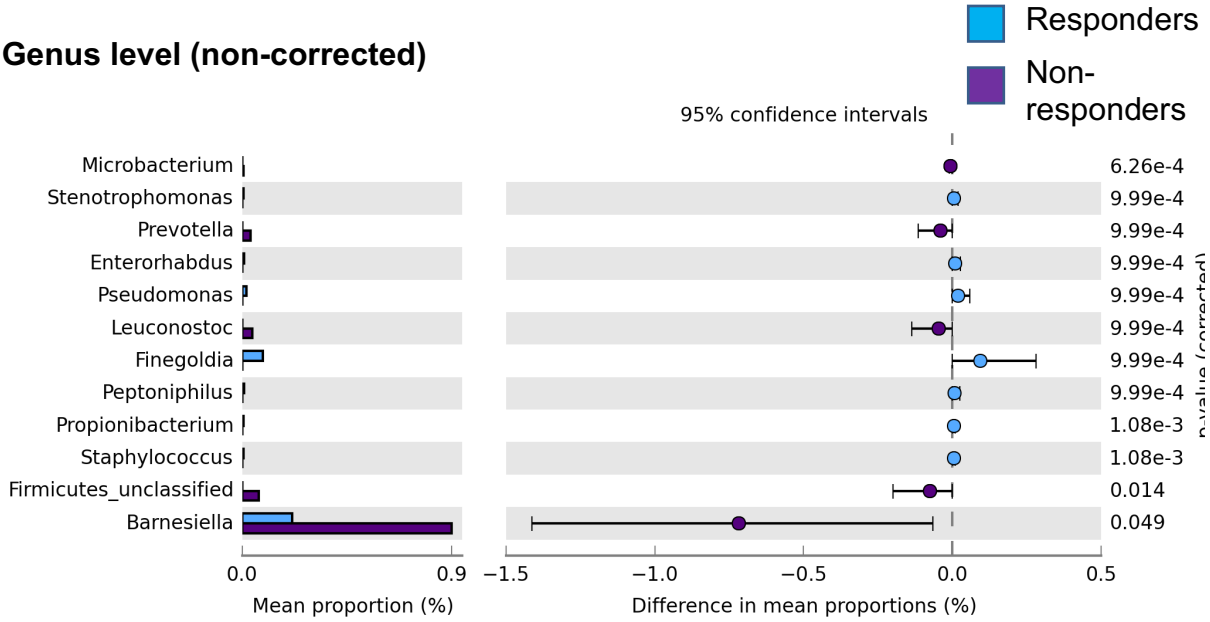
samples. There were 10 responders and 13 non-responders. Due to logistical reasons 25 healthy controls and 10 paired samples were lost during the duration of the study.

Table 17. Antibiotics used in cohort

Ciprofloxacin and Metronidazole	4
Cefuroxime	1
Doxycycline	1
Metronidazole	1
Co-amoxiclav and ciprofloxacin	1
Ciprofloxacin	2
Co-amoxiclav	2
Total	12

When comparing responders on antibiotics vs non-responders off antibiotics and responders off antibiotics with non-responders off antibiotic there were no significant differences across all taxonomic levels. We therefore looked at any differences between responders and non-responders

Genus level (non-corrected)



Order level (non-corrected)

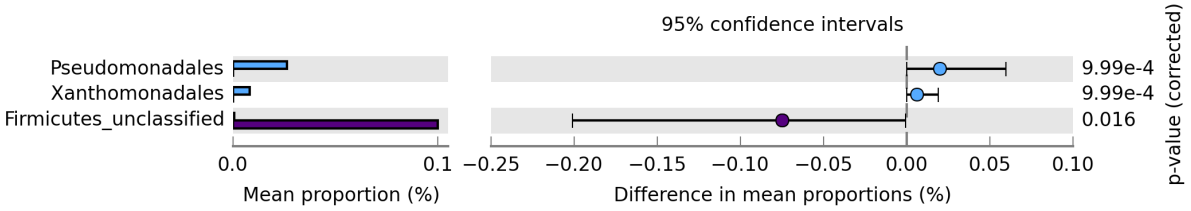
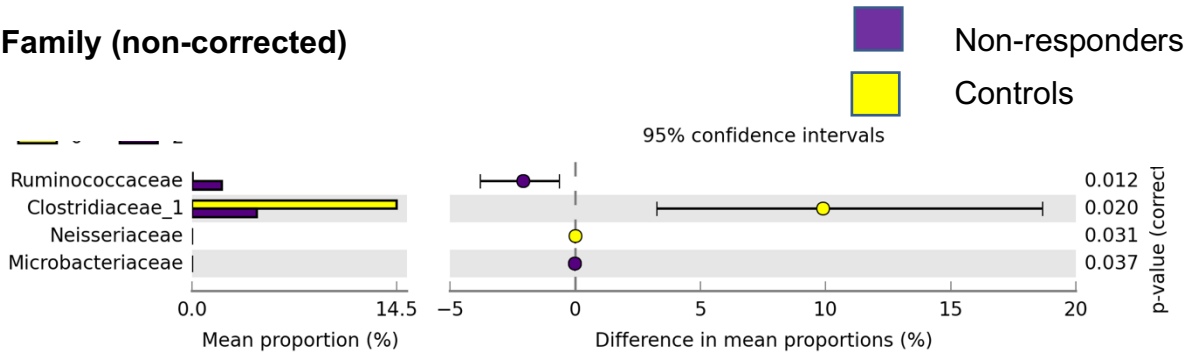
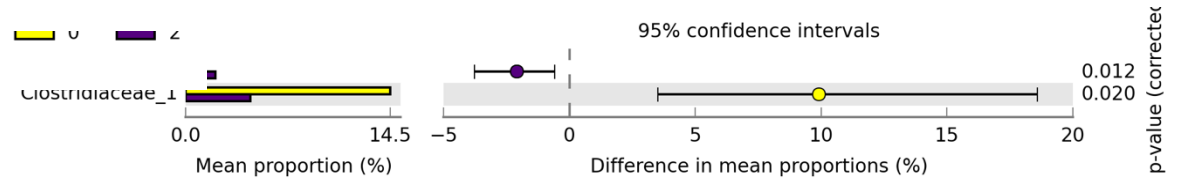


Figure 19. Differences between responders and non-responders

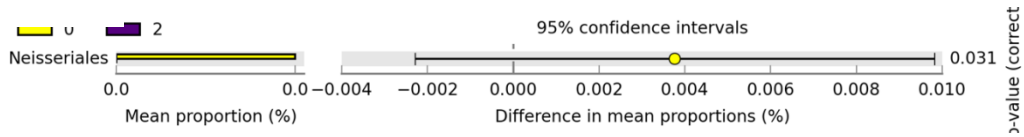
Family (non-corrected)



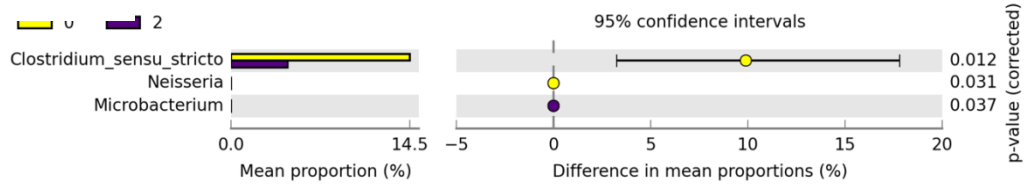
Family (after-correction)



Order (non-corrected)



Genus (non-corrected)



Genus (corrected)

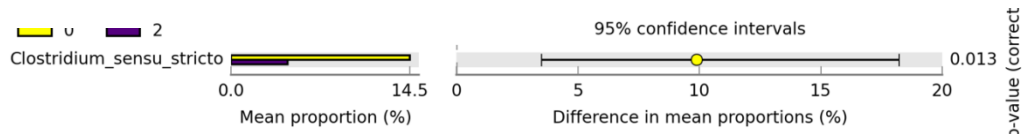
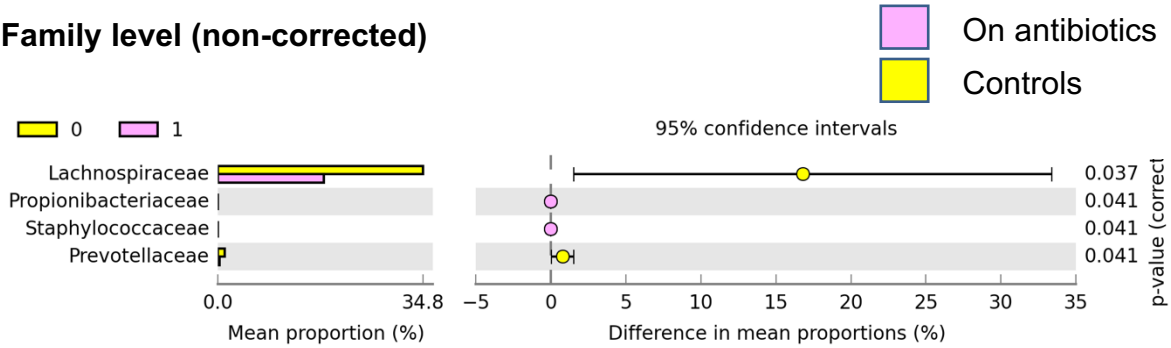
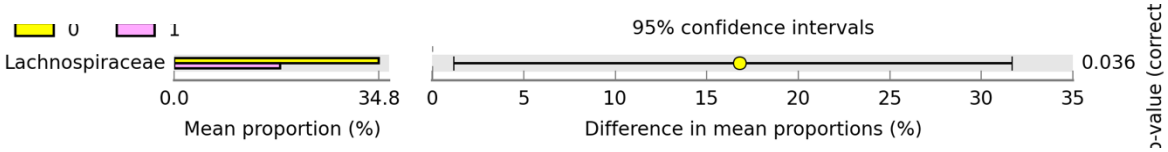


Figure 20. Differences between non-responders and controls

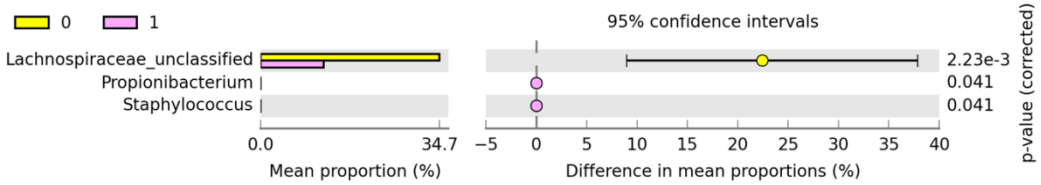
Family level (non-corrected)



Family level (after correction)



Genus level (non-corrected)



Genus level (after correction)

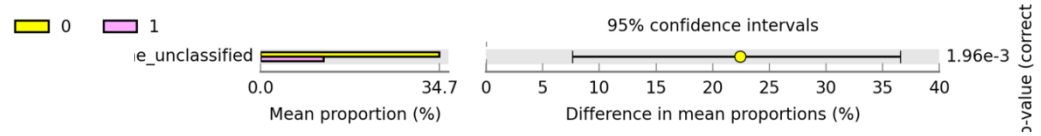
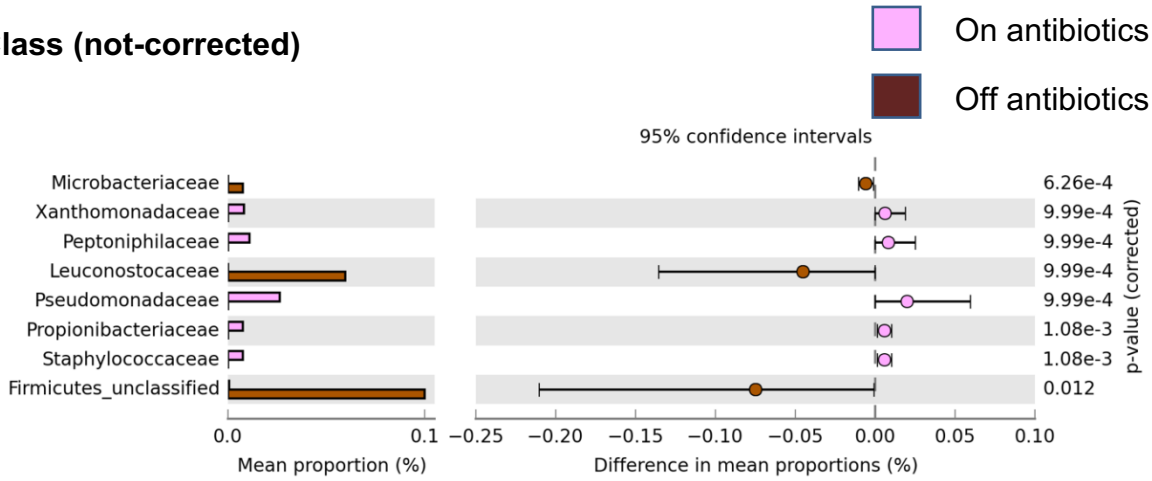
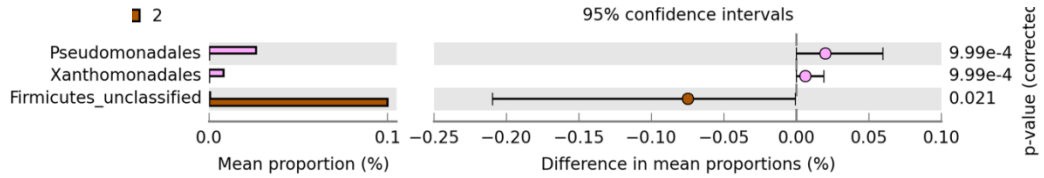


Figure 21. Difference between those on antibiotics and controls

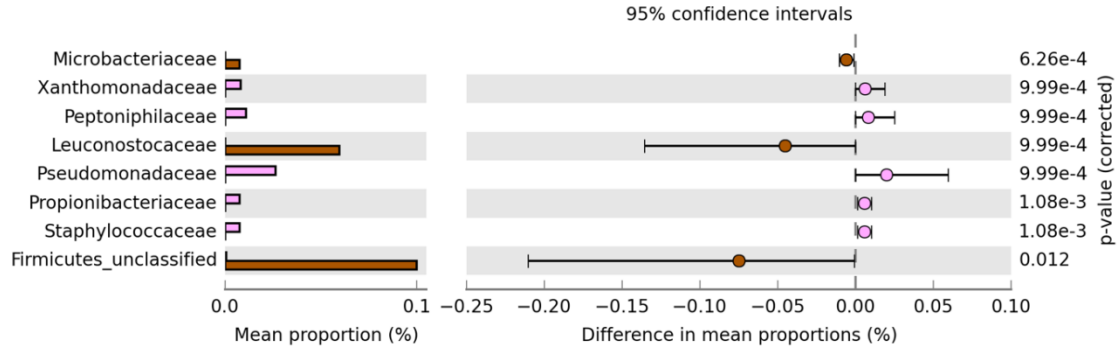
Class (not-corrected)



Order (not-corrected)



Family (not-corrected)



Genus (not-corrected)

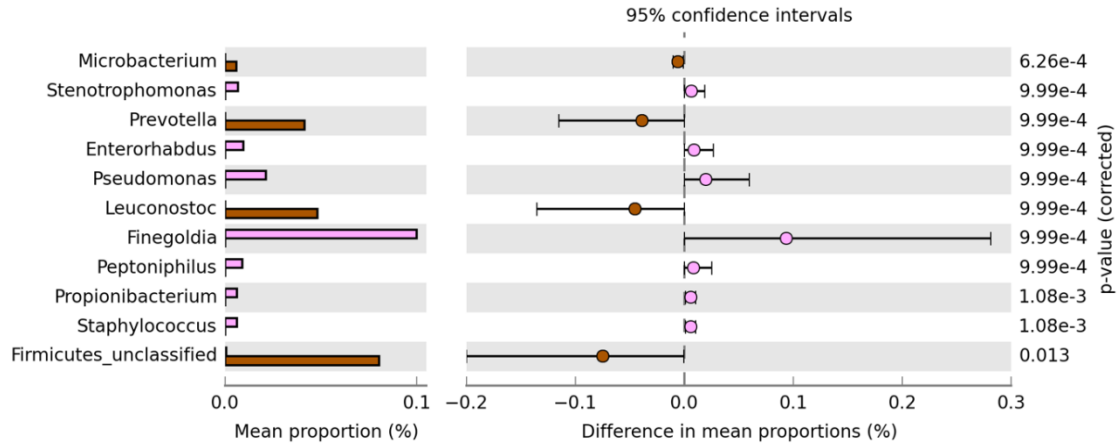
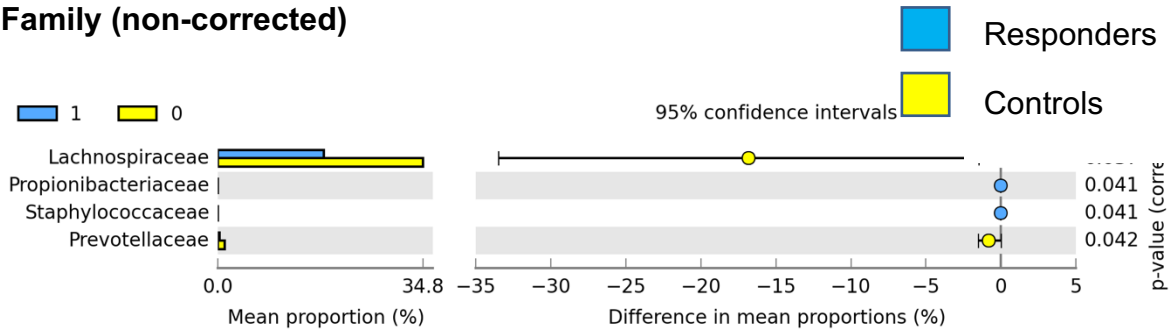
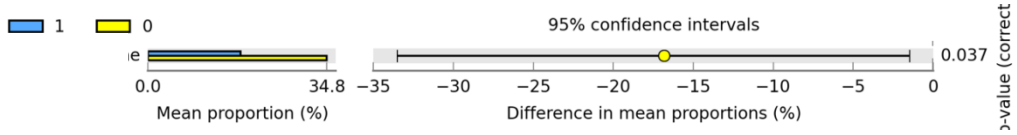


Figure 22. Differences observed between subjects on and off antibiotics.

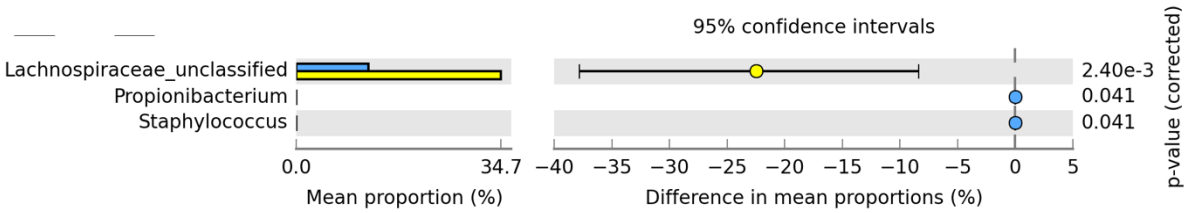
Family (non-corrected)



Family (after correction)



Genus non-corrected



Genus (after correction)

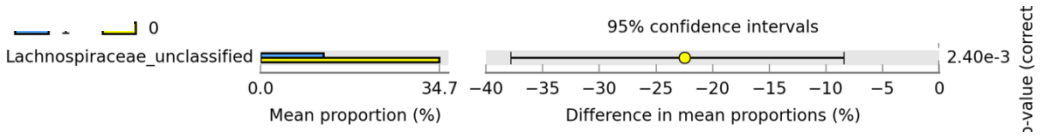


Figure 23. Differences observed between responders and controls.

Table 18. Corrected Q values of significant findings.

Comparison	Taxonomic level	Increase observed	Change observed	Q value
non-responder vs control	family	increased controls	ruminococceae	0.01
non-responder vs control	family	increased controls	clostridiaceae	0.02
non-responder vs control	genus	increased controls	Clostridium sensu stricto	0.01
responders vs controls	family	increased controls	lachnospiraceae	0.03

Comparison	Taxonomic level	Increase observed	Change observed	Q value
responders vs controls	genus	increased controls	lachnospiraceae unspecified	2.23×10^{-3}
on antibiotics vs controls	genus	increased controls	lachnospiraceae unclassified	2.14×10^{-3}

Figure 24. Heat map Family level non-responders vs controls.

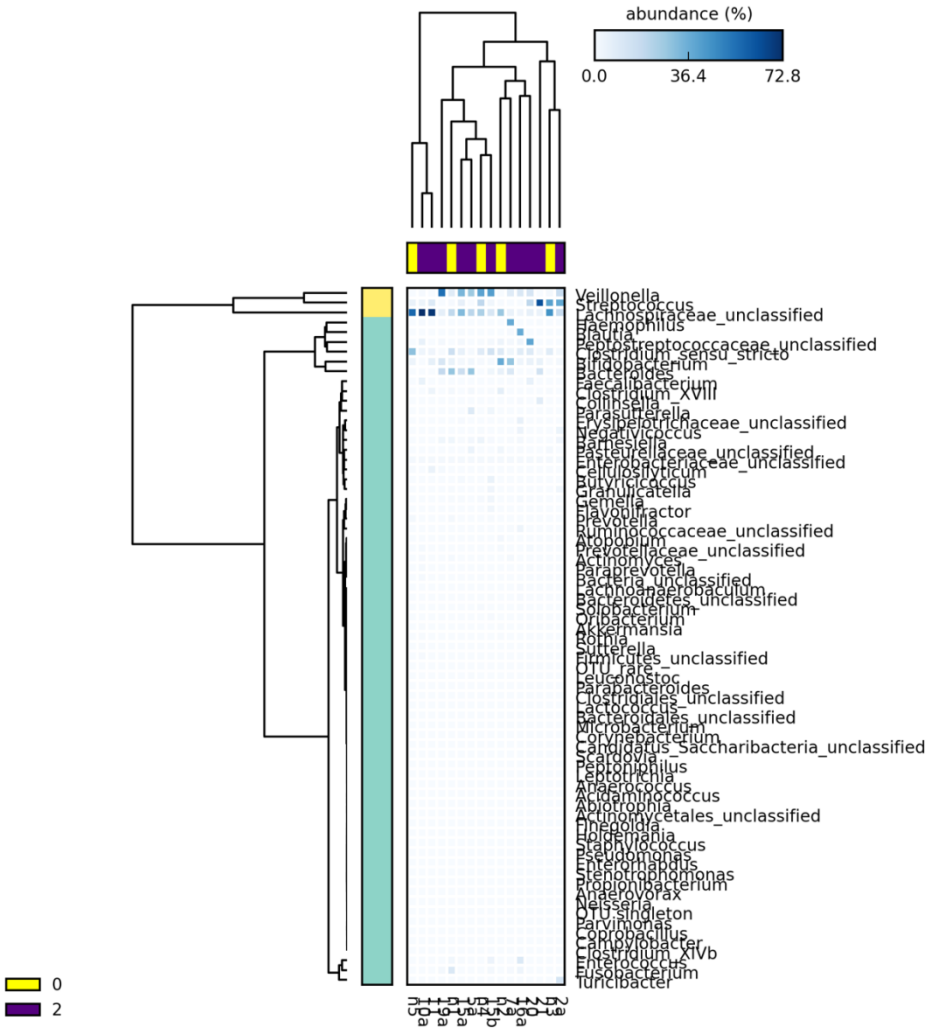


Figure 25. Heat map Genus level non-responders vs controls.

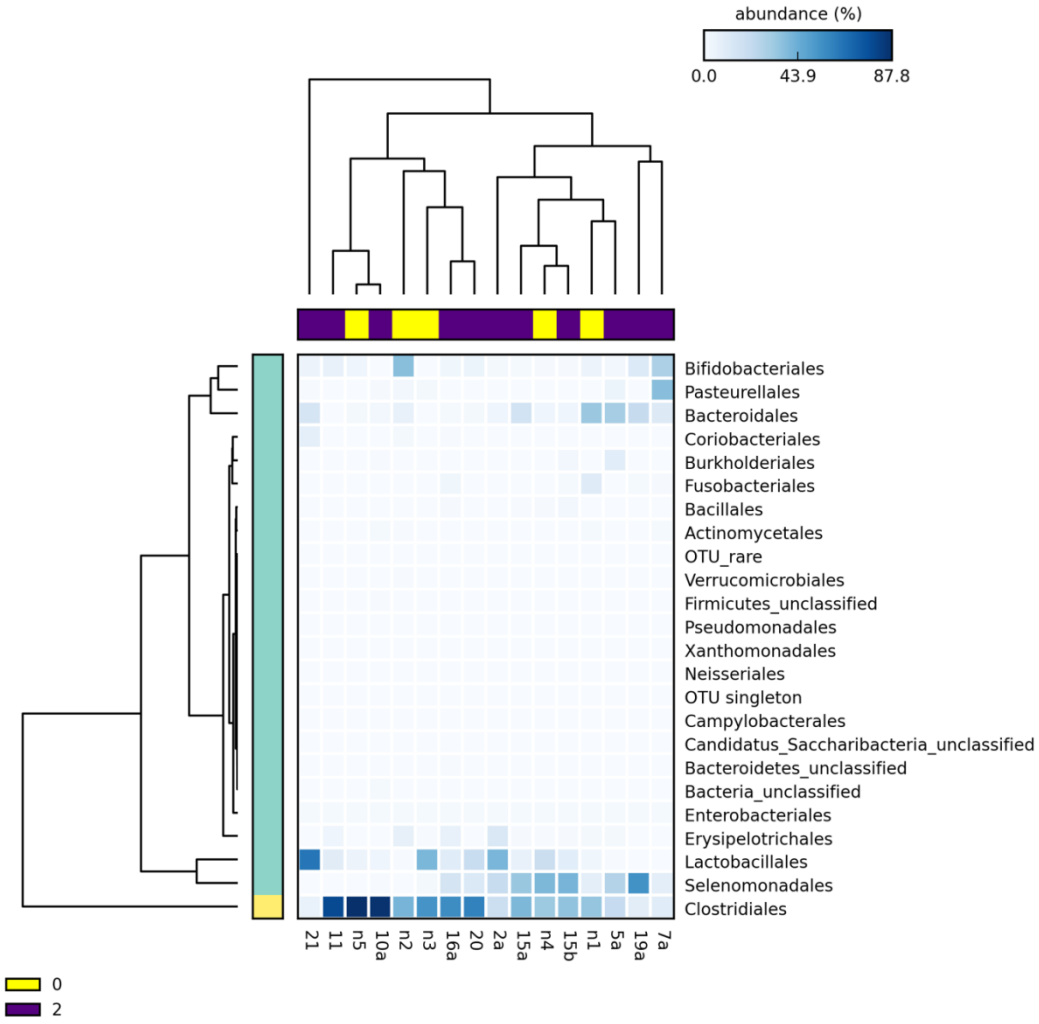


Figure 26. Heat map Order level non-responder vs controls.

Table 19. Shannon-diversity index responders vs non-responders vs controls.

	Responder	Non-responder	Control
Mean	1.3661318	1.54721777	1.6198704
Median	1.4662975	1.7482105	1.752349
Range	(0.224211-1.977959)	(0.33287-2.802115)	(1.129616-2.239235)

Table 20. Shannon diversity index on vs off antibiotics vs controls.

	On antibiotics	Off antibiotics	Control
Mean	1.37322142	1.71808573	1.7253282
Median	1.188854	1.808474	1.752349
Range	0.224211-2.802115	1.147994-2.231835	1.129616-2.239235

Table 21. Shannon diversity index responders vs non-responders on antibiotics.

	Responders on antibiotics	Non-responders on antibiotics	P value
Mean	1.119256	1.36011167	0.60
Median	1.188854	1.3559635	
Range	0.224211-1.733846	0.33287-2.802115	

Table 22. Shannon diversity responders vs non-responders off antibiotics.

	Responders off antibiotics	Non-responders off antibiotics	P value
Mean	1.7364455	1.70759443	0.90
Median	1.827459	1.808474	
Range	1.312905 -1.977959	1.23608-2.231835	

Table 23. Shannon diversity comparison between groups using parametric testing.

Comparison	P value
Responder vs non-responder	0.50
Responder vs control	0.37
Non-responder vs control	0.80
On vs off antibiotics	0.44
On antibiotics vs control	0.22
Off antibiotics vs controls	0.69

The above table highlights that there were no significant differences in alpha diversity (Shannon diversity) between the groups. This is represented graphically below:

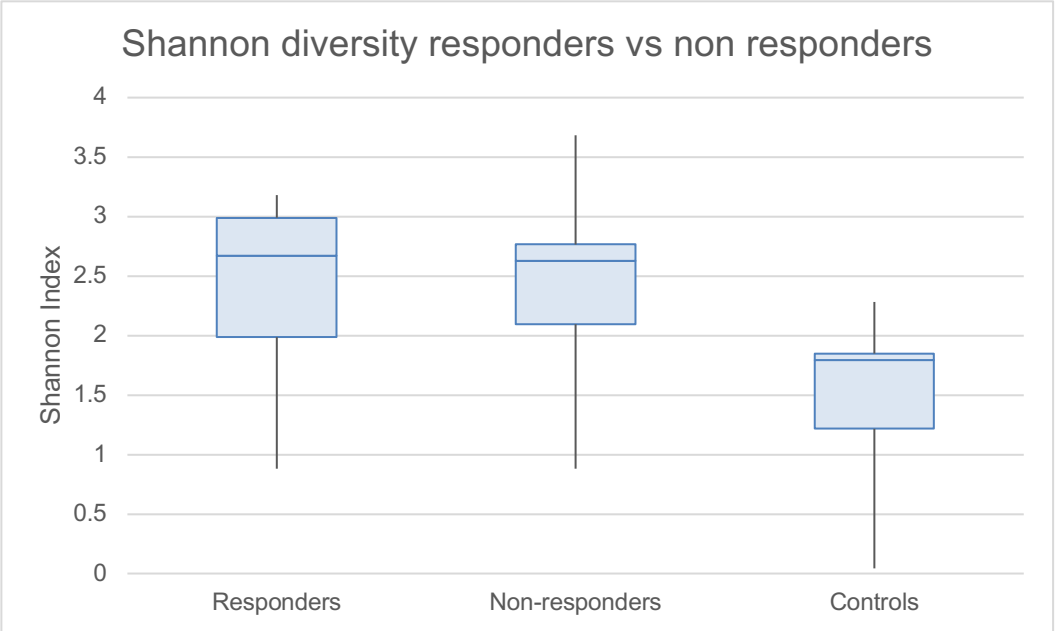


Figure 27. Shannon diversity responders vs non-responders.

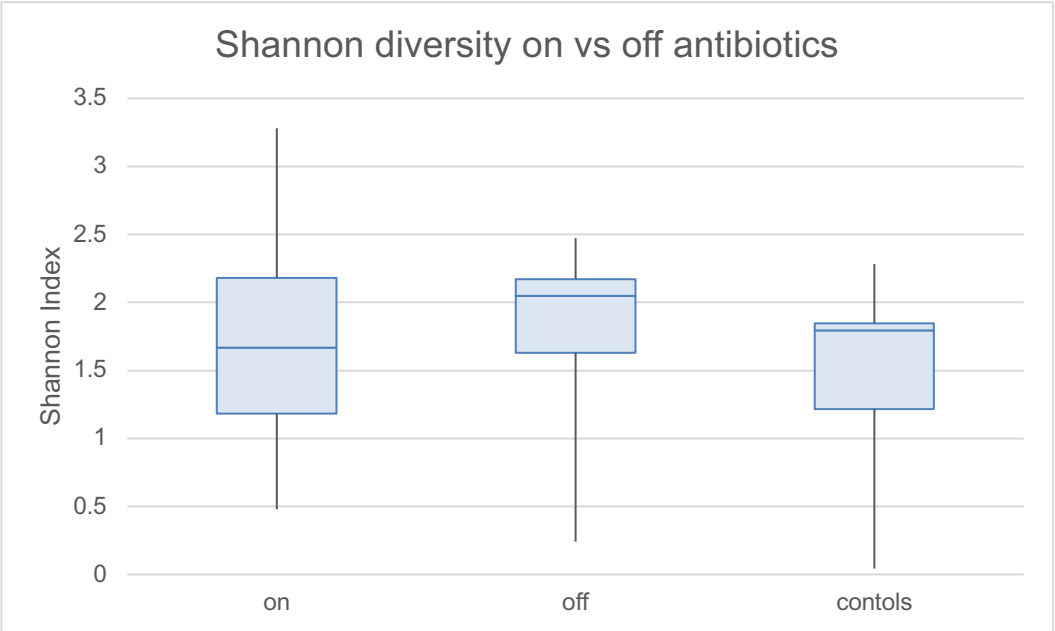


Figure 28. Shannon diversity on vs off antibiotics vs controls.

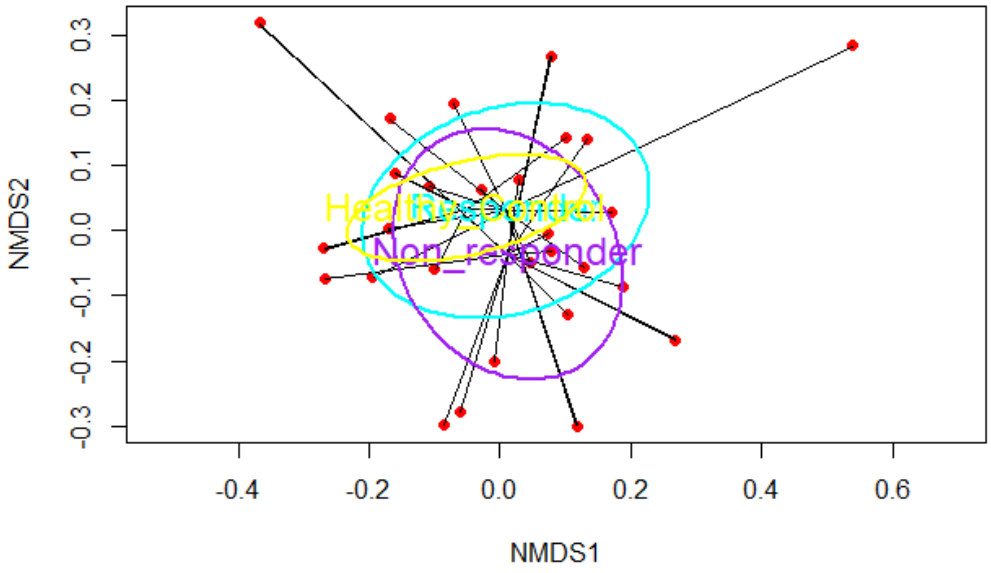


Figure 29. NMDS Plot Responder vs non-responder vs control.

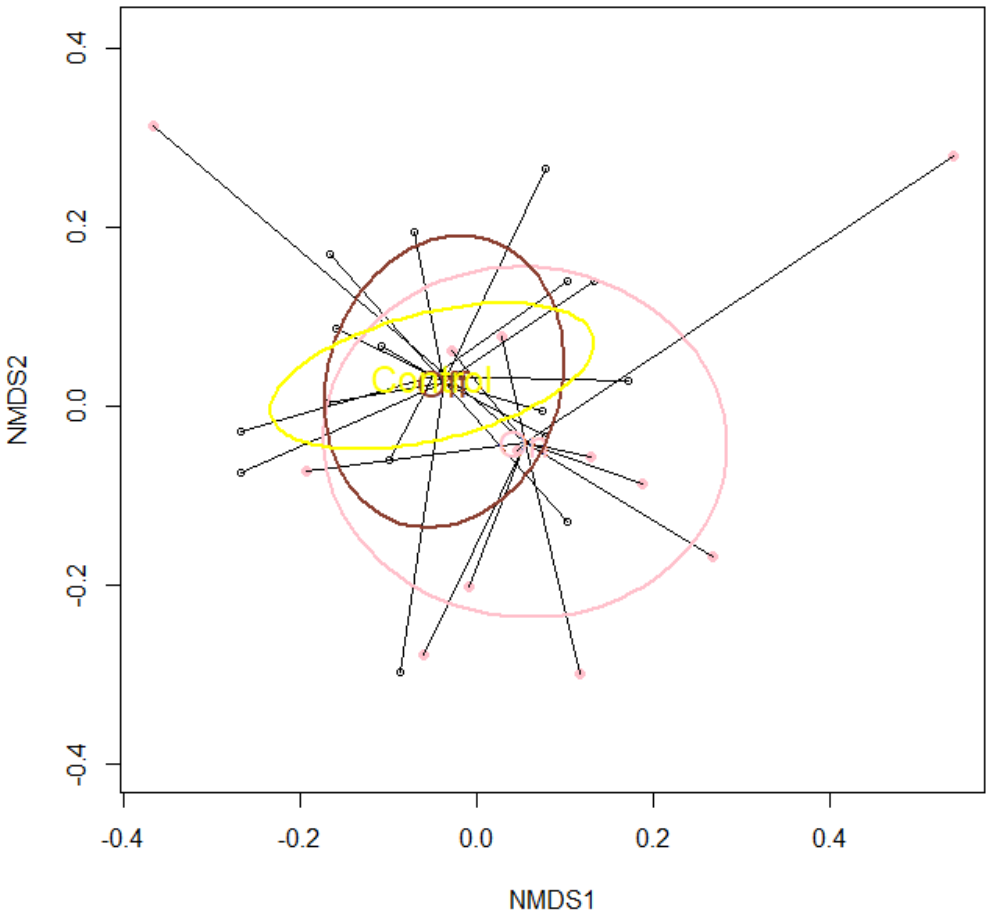


Figure 30. NMDS plot on antibiotics vs Off antibiotics vs control.

Table 24. Permanova comparison.

Comparison	F.model	R2	p-value	Corrected p-value
Responder vs non-responder	1.2968175	0.0581655	0.24	0.35
Responder vs healthy control	0.3336155	0.02502034	0.92	0.92
Non-responder vs healthy control	1.8419942	0.10323926	0.11	0.34

Table 25. Permanova comparison.

Comparison	F.model	R2	p-value	Corrected P-value
On vs off antibiotics	1.3671263	0.06112212	0.237	0.36
Controls vs off antibiotics	0.6404766	0.04373698	0.688	0.69
On antibiotics vs controls	1.4994475	0.09087865	0.181	0.36

10.1.4 Discussion

These data demonstrate that healthy controls have significantly higher proportions of *clostrostrium* genera when compared with patients who fail to respond to antibiotics. Furthermore, healthy controls have significantly higher proportions of *lachnospiraceae* species when compared with those that responded to antibiotic treatment following PIP. Although not statistically significant, there was a higher proportion of Firmicutes in the group off antibiotics compared to those that were on antibiotics.

Interestingly, both alpha and beta diversities were not altered across the different groups suggesting that perhaps antibiotics and its effect on the pouch faecal microbiota are not driving the clinical effects. This is further supported by the lack of significant differences found between responders and non-responders. Reasons for the lack of difference between patients on and off antibiotics and responders vs non-responders are likely multifactorial to include small samples size, lack of control for diet and the underlying disease itself. It has been shown that in certain patients chronic PIP is persistent and unresponsive to treatment[216]. It is therefore possible that

clinical response is transient in chronic PIP and as such the microbiota signatures do not change significantly. Furthermore, the tool used to determine response, the PDAI is not validatated. It is therefore possible that this tool is not sensitive enough to determine differences in microbiota patterns between responders and non-responders. Another potential reason for lack of microbiota differences may be on the relative fast transit of the faecal stream in a pouch. It is possible that the fast transit does not allow engraftment of specific bacteria onto the stool and mucosa and therefore changes not observed. Furthermore, many of the previous studies observing changes in the microbiota have been observed in acute PIP[200,306]. It may well be that the chronicity of chronic PIP means that the microbiota becomes developed and less responsive to change.

A significant potential reason for the differences we found from the current literature in terms of lack of change in diversity is the differences in technologies used in this study to observe microbiota patterns[200,306]. With next generation sequencing techniques such as the one used in this process, it was possible to detect bacterial communities such an anaerobic bacteria that may be under-represented by techniques such as culturing techniques. Other studies reporting the microbiota in the pouch are also based on small numbers[306].

Whereas there are clear differences between these data and the established literature, these findings are in keeping with the possible importance of clostridia as highlighted in my systematic review where it was shown that patients with chronic PIP have lower levels of clostridia species.

There are many limitations in the study, mainly lack of control for major confounding factors such as diet, other medications and lifestyle factors. It is likely that these had an impact on the microbiota. Furthermore, the study is limited by small numbers which reflects the difficulty in recruiting patients with a rare disease and a rare complication.

Future studies should attempt to build on this work with larger numbers, where possible dietary factors should be adjusted for as well as lifestyle differences such as smoking and medication use.

In terms of the potential mechanistics behind these associations, it has been shown that *Clostridium* species are important in maintenance of gut health and production of

butyrate[337]. Lachnospiraceae has been associated with acrylate pathway that produces proprionate[338]. Furthermore it has been shown that Firmicutes decreased in PIP, responsible for butyrate production[206]. A unifying theme therefore suggests that the bacterial populations producing SCFA are protective against PIP and the removal of them may lead to inflammation. It may also be possible that the effects of these SCFA are occur on a tissue level and therefore may poorly correlate with faecal SCFA levels.

Taking this work forward it seemed evident that it is not just the gut microbiota that is driving the disease process. It is therefore possible that the metabolic activity of some of the microbiota may contribute to some of the disease process. The next chapter therefore tries to explore if there is a link between a common metabolic by-product short chain fatty acids that have been linked to inflammation in IBD.

10.1.5 Conclusion

A healthy pouch has significantly higher proportions of clostridia species, the importance of this has been supported in other literature. Despite these findings, there are few consistent microbial patterns that can differentiate between responders and non-responders to treatment.

An important consideration is understanding if the inflammatory process causes the change in the microbiota, or vice versa. To help determine this, functional studies are required to look on a deeper level regarding changes that may occur in conjunction with the alterations in the microbiota. Furthermore, in the inflammatory process, it may be that alterations in bacterial metabolic capabilities are more important than individual bacterial changes. Therefore, looking at functionality of the ensuing microbiota rather than trying to define the community composition may allow a better understanding of the microbiota's role in IBD. The next chapters explore the metabonomic pathways that may be associated with inflammation in the pouch.

Chapter 11

Nuclear Magnetic Resonance (NMR)

11.1 Introduction to NMR Spectroscopy

NMR spectroscopy utilises the properties of nuclei in a magnetic field which absorb and then re-emit electromagnetic radiation. The energy emitted is at a specific resonance dependent on the strength of the magnetic field and the magnetic properties of the isotope present. This allows structural and quantitative information to be gathered on hundreds of metabolites in a biofluid sample.

11.2 Basic concepts of NMR spectroscopy

11.2.1 ^{1}H NMR Chemical shifts

Each chemically distinct nucleus in each metabolite within a biological sample will exhibit a NMR signal which corresponds to a specific resonance frequency which is measured relative to a known compound and is called the chemical shift[339]. In many NMR experiments the compound 3-(trimethylsilyl)-2,2′,3,3′-tetradeuteropropionic acid (TSP) is used as a reference material and has the resonance of 0 parts per million (PPM).

The determinants of a chemical shift of an NMR signal depends on the hydrogen nucleus in a metabolite and is independent of the applied field strength. Furthermore the signal is highly reproducible and precise for that nucleus[339].

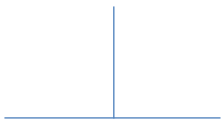
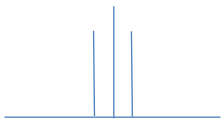
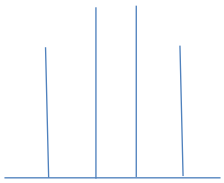
11.2.2 ^{1}H NMR Multiplicities

Multiplicity refers to the pattern of peaks that is observed for a particular hydrogen signal. The multiplicity indicates how many hydrogen atoms are immediately next to the hydrogen atoms that are responsible for that peak. The importance of multiplicity is it allows identification of the NMR spectrum. Multiplicity follows the N+1 rule, as an example, the methyl (CH_3) in the following example: **CH_3**CL has no neighbouring hydrogen atoms and would therefore appear as a singlet. if this methyl group had one

hydrogen next to it as in the example **CH_3**$CHCl_2$, the hydrogen would split the signal from the methyl group into two peaks (doublet). The following compound CH_3CH_2CL with three neighbouring hydrogen atoms would therefore split the methyl group into three peaks called a triplet. Beyond this we refer to peaks with more than three neighbouring hydrogens as a multiplet.

A summary is shown below

Table 26. Summary of splitting patterns.

High Resolution NMR	Described as	Caused by how many H neighbours
	Singlet	0
	Doublet	1
	Triplet	2
	quartet	3

11.2.3 Pre-processing NMR data

Free induction decay (FID) is the observed NMR signal that is generated by non-equilibrium nuclear spin in magnetization changing orientation of a nucleus around an axis about a magnetic field (conventionally along z)[340]. Zero filling then manipulates this FID to increase the digital resolution in the spectrum. Following this apodisation multiplies the FID to optimise the quality of the spectrum. Fourier transformation then provides a short pulse of frequency aimed at the centre of the spectra with the aim to incorporate contributions from all the frequencies in the neighbourhood of the principal frequency[341]. Raw information generated from analysis of the samples needs to be adjusted to correct for artefacts. Baseline correction involves adjusting the baseline to

account for ‘noise’ that is generated by a persistent generation of positive and negative signals which are assumed to average around zero[342]. This noise may create broad regions of peaks that do not contain any signal of interest and therefore require correction. A distorted baseline may alter intensity of spectra of interest and therefore decrease the accuracy of spectra identification. Furthermore many multivariate statistical analyses cannot differentiate between distorted baselines and spectra[343]. In NMR analysis frequency domain baseline correction a common approach and it involves baseline estimation and its subsequent subtraction directly from the measured spectrum[344].

11.2.4 Peak alignment

A significant challenge in NMR is dealing with the chemical shifts of peaks which can be caused by pH, temperature, ion content and instrument factors[345]. Methods to partially avoid this include using buffer solutions in an attempt to stabilise the pH[346]. The risk of not aligning the data means that potentially properly matched and downstream univariate or multivariate quantitative analysis of their signal intensities can be compromised[345]. There are various methods to align spectra which are summarised well by Vu *et al*[345].

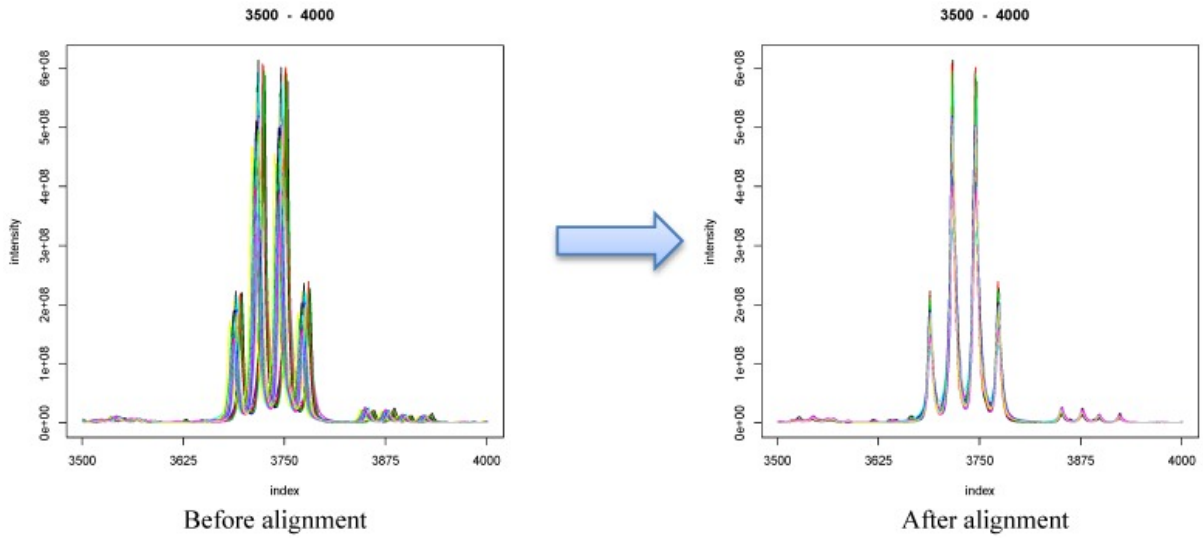


Figure 31. Example of peak alignment taken from Vu el al [345].

Reproduced with permission from [345]

11.2.5 Scaling

Scaling is performed on each spectral intensity across samples. This helps analyse the data. Scaling is done so that components used in the modelling analysis have as their origin the centroid of the data, resulting in a parsimonious model[347].

11.2.6 Normalisation

The aim of normalisation is to allow sample to be directly comparable to each other. The aim of this process is to try to limit the variable dilution of the samples. Where there is tight homeostasis in certain biofluids such as serum this is less of an issue. In samples such as urine a large variation exists with contributions from drugs, pH and urinary volume.

11.2.7 2D 1H J-resolved (JRES) NMR spectroscopy

The JRES is a method to aid the identification of the NMR spectra[348][349]. JRES is a 2D homonuclear experiment that plots the chemical shift along one axis and the proton-proton coupling on the other [348][349]. This allows for easier identification of the multiplicity of the NMR spectra. An example is shown below

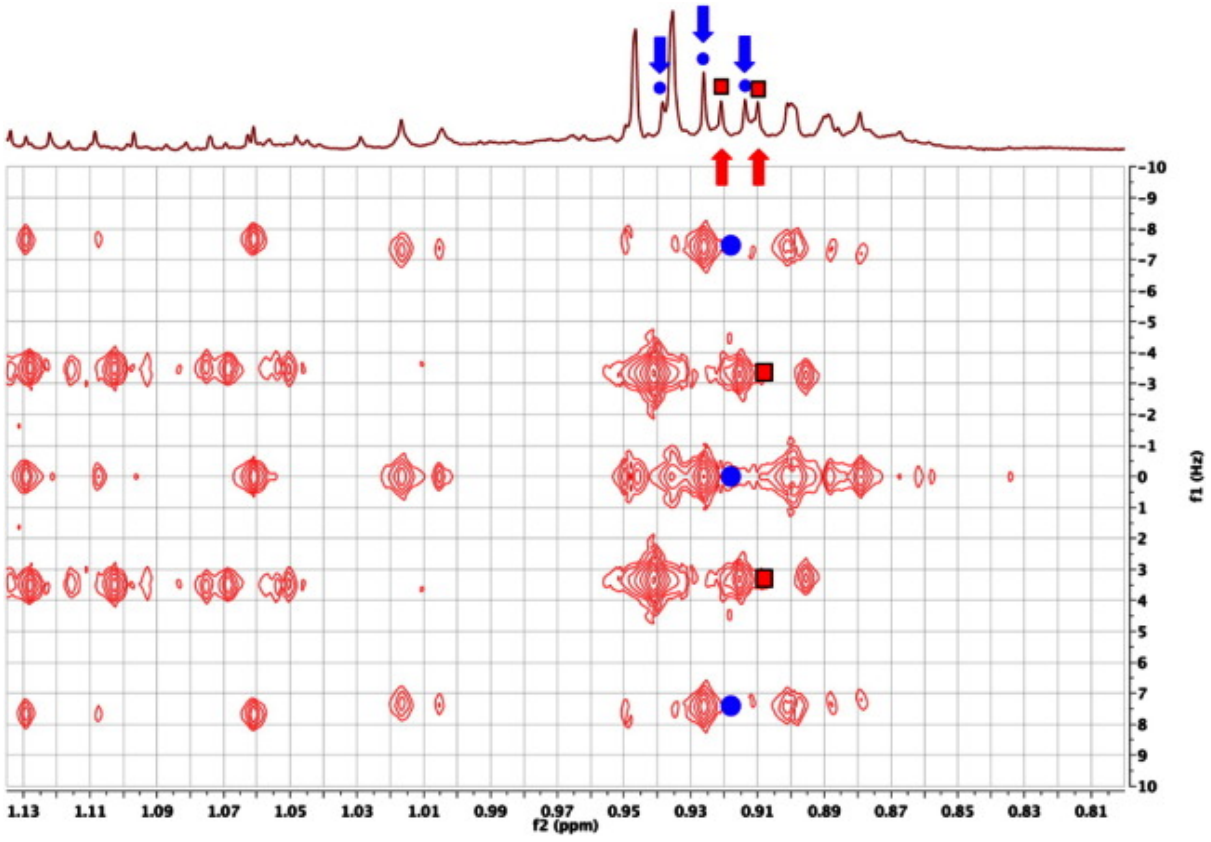


Figure 32. 2D 1H J-resolved (JRES) NMR spectroscopy.

Reproduced with permission from[350]. The blue dots representing the triplet methyl group of N-butyrylglycine (0.916ppm) and the doublet the methyl group of isovaleric acid (0.916ppm).

11.2.8 Statistical Models used

NMR data generates large multivariate data-sets. In order to discern meaningful patterns from the dataset and identify metabolic signatures that may aid disease prediction or diagnosis broad range of statistical methods can be used. The most common of these are unsupervised models which include principle component analysis (PCA)[351]. Supervised approaches include partial least squares (PLS)[352], partial least squares discriminant analysis and orthogonal partial least squares discriminant analysis (OPLS-DA)[353].

The PCA model is the most common technique used for NMR and multivariate analysis. It works by providing an unbiased overview of the variability in a dataset by reducing dimensionality[354]. This model clusters samples based on their similarities and differences without any prior knowledge as to what group the sample belongs to. PCA works by representing variance within a dataset by utilising principle components (PCs). Each PC is a weighted linear weighted combination from the original variables and is statistically independent to the previous PC. By doing this it describes the maximum additional variation in the data that is not accounted for by the previous PCs[354]. PCA plots allow for visualisation of groupings along with trends and outliers. The PCA plots also provide ‘loadings’. These demonstrate which variables are providing the biggest contributions to the positions of the samples found on the plot. Importantly the direction of the loadings correspond to the position on the plot and therefore an understanding of the loadings can help explain the clustering found on a PCA plot[355,356].

Following PCA analysis a supervised model is then undertaken. Unlike PCA models these models use groupings of samples to enable maximum separation between the groups in order to try and identify the metabolic patterns that may contribute to the classifications[354].

The most commonly used supervised model is the PLS model. This model links spectral values which are labelled as ‘x values’ with qualitative values that help separate the groups labelled as ‘y values’. When the y matrix contains categorical information, the PLS model is called partial least squares- discrimination analysis (PLS-DA)[354].

A further supervised model called the orthogonal partial least squares discriminant analysis (OPLS-DA) which excludes irrelevant data or data that are uncorrelated which can sometimes be referred to as ‘structural noise’[354]. In biological samples this ‘structural noise’ can be considered part of physiological variation and may include diet, age, gender, medications etc. This model attempts to remove irrelevant variation and therefore may enhance the observed clustering of different samples.[357]. These supervised methods use loadings to provide weighting, variable importance on projection (VIP) and regression co-efficient plots which help determine the variables that are having the most influence on the model[354].

The limitations of supervised models are they can be prone to overfitting especially where there are a large number of variables. This has potential for forcing models and creating a false model. It is therefore important to use cross validation.

11.2.9 Metabolite identification

Metabolite identification involves using the multivariate models described above to help detail which NMR signals are responsible for significant differences observed. It is these significant signals on which identification is attempted. Identification involves finding out the multiplicity of the metabolites (as described above) then using statistical software to highlight correlated peaks. Using databases such as the Human metabolome database and published literature[358], peaks can be identified.

11.2.10 Statistical correlation spectroscopy for metabolite identification (STOCSY)

STOCSY is a statistical method used to help identify peaks that are found to be significant from the supervised models. STOCSY utilises the inherent linear relationship between intensity variables that belong to the same molecule in an NMR spectrum[339]. By analysing the covariance of variables it can produce degrees of correlations between variables. STOCSY provides information about how strongly

correlated a peak is using an R^2 value. The stronger the association the closer the R^2 value is to 1[359]. To help with this, the correlations are often colour coded. The values correlated that are closer to 1 are likely to derive from the driver peak. The use of STOCSY is especially important in the analysis of complex biofluids such as urine where there is considerable overlap between resonances[359]. The degree of overlapping of a sample can affect the ability of STOCSY to correlate samples as it distorts the covariance. Other techniques such as SubseT Optimization by Reference Matching (STORM) have been developed to help with this[360].

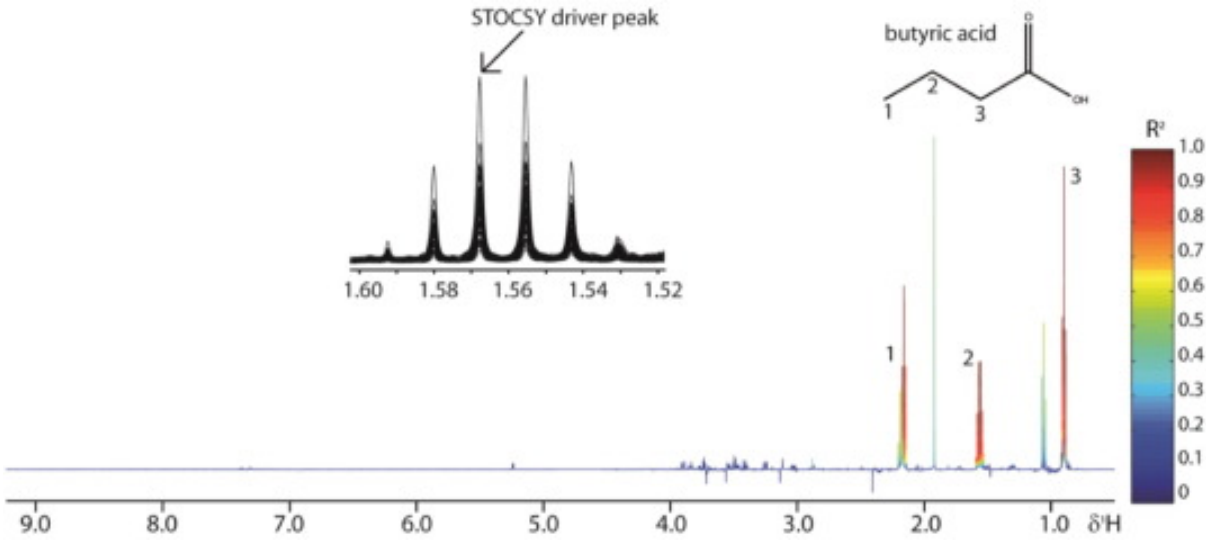
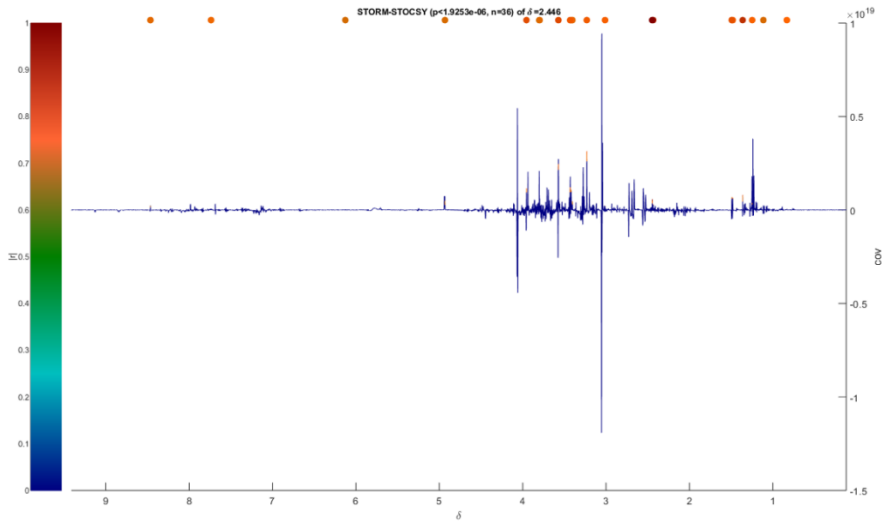


Figure 33. Example of Stocsy.

Reproduced with permission from [339]. The driver peak is the arrow at PPM 1.57. this corresponds to butyric acid. The graphic to the right of this demonstrates the correlated peaks that arise from the driver peak and therefore are likely to arise from the same molecule.



11.2.11 STORM

STORM was developed to select subsets of (1)H NMR spectra that contain specific spectroscopic signatures of biomarkers differentiating between different human populations[360]. STORM tries to distinguish low-intensity and rare signals close to the baseline from noise in the plot by finding a subset of spectra that contain the purest form of the unknown signal[360]. STORM learns a true reference of a spectra by finding the most correlated spectra to a driver peak and re-running multiple further correlations based on the most correlated peaks[360]. It learns the true reference by repeating a procedure to find the most highly correlated spectra and updating the reference multiple times

11.3 Introduction to NMR study

The aetiology of PIP is poorly understood but is thought to involve the complex interplay between the host genetics, the microbiota, immune system and environment[361]. To date mechanistic work regarding the aetiology of PIP has focussed on the gut microbiota[206], genetic and immunological functions[139,140,362] including cytokines[363] but few data exist on the metabolic contribution these may have to PIP

Specifically, in acute PIP *Clostridium* species are increased[309,314,318] with decreases in *Enterococcaceae*[317][320]. On a genetic level PIP has been

associated with NOD 2insC variant[364]. Currently these remain all associations with PIP and require further validation in larger studies.

Metabonomics is “the quantitative measurement over time of the metabolic responses of an individual or population to drug treatment or other intervention” [365]. Its advantage is using an integrated systems biology approach which provides a way of investigating the metabolic status of an organism or ecosystem but studying “real” metabolic endpoints[366].

Metabonomics can be used to predict responses to medical treatment termed pharmacometabonomics and can be measured when samples are collected and analysed both prior and after a medication is given [367] as well as be used to predict disease states with the potential for personalised medicine[368][369]. This integrated technology utilises many instruments, with the main ones being ^{1}H-Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS), which is split into Liquid Chromatography-Mass Spectrometry (LC-MS) and Gas Chromatography-Mass Spectrometry GC-MS. The integration of chemometrics then helps understand the metabolities which profile the metabolites with the potential to use them to predict disease vs non-diseased states.

Metabonomics therefore enables profiling the end product or metabolite found in biofluids. This can enable longitudinal assessment of metabolic changes, metabolic changes in response to treatment and metabolic profiles in both healthy and diseased states. Metabonomic profiling can provide insights into unique fingerprints of biochemical perturbations that is characteristic of the nature or site of toxic insult or disease process[370]. This can therefore be the basis of finding novel biomarkers such as the (R)-2-hydroxygularate which is a biomarker of gliomas and acute myeloid leukaemias[371].

Metabonomics can link metabolites found to specific metabolic pathways which can then directly be correlated in with the bacterial metabolic pathways and therefore advance the interplay of the microbiota and metabolic pathways on disease aetiology.

Metabonomics is a relatively new technique but has been utilised in inflammatory bowel disease in an attempt to elucidate its mechanistic aetiology. Williams *et al,* using NMR profiling of urine, found that significant decreases in Hippurate in IBD patients

when compared to healthy controls[372]. When looking at serum, differences have been shown between amino acids and TCA cycle molecules between ulcerative colitis (UC) and Crohn's disease patients (CD) [373,374]. Marchesi *et al* have highlighted that faeces from IBD patients showed lower level of short chain fatty acids (SCFA) when compared with healthy subjects through NMR profiling[375]. Specifically, they found that a depletion in the SCFA including acetate and butyrate in patients with CD when compared with healthy controls. Tissue is another source of material that can be analysed using omics. It is one of the less studied. Sharma *et al*., highlighted that the metabolic profile of colonic amino acids membrane components and lactate were similar between non inflamed IBD segments and inflamed segments[376] Bjerrum *et al* highlighted that colonic biopsies from patients with active UC had higher levels of antioxidants and of a range of amino acids, but lower levels of lipid, glycerophosphocholine (GPC), *myo*-inositol, and betaine when compared with healthy controls.

NMR analysis is one of the methods discussed in the introduction that can provide a metabolic profile. NMR analysis may therefore highlight unique biomarkers of PIP whilst highlighting potential mechanistic pathways for the development of PIP. This chapter describes the use of NMR to examine changes longitudinally in a pouch (study 1) and to investigate if differences exist in health and disease states (study 2).

11.3.1 Hypothesis

Study 1

1. There will be significant metabolic differences observed by NMR between ulcerative colitis patients and familial adenomatous polyposis patients.
2. There will be significant longitudinal metabolic differences observed by NMR between those that develop PIP and those that do not develop PIP.

Study 2

1. There will be significant metabolic differences observed by NMR between responders to antibiotics and non-responders to antibiotics.
2. There will be significant metabolic differences observed by NMR between those with PIP and healthy controls.

11.3.2 Methods

11.3.2.1 Patients recruitment

For both studies, patients above the age of 16 years who were being considered for restorative proctocolectomy (RPC) were eligible for the study. Patients were excluded if they were unable to provide written consent, were pregnant at the time of consent or had undergone previous restorative proctocolectomy.

For both studies ethical approval was granted by the Brent Research Ethics Committee **ID:08/H0717/24: Prospective study of immunological and microbiological factors in inflammatory bowel disease.**

11.3.2.1.1 *Study 1 design: longitudinal cohort*

Patients were recruited using a prospectively maintained pouch database at a single institution which highlighted potential candidates for restorative proctocolectomy. Patients who agreed to undertake the study were then reviewed and consent was obtained.

Twenty patients were enrolled and provided early morning mid-stream urine both prior to undergoing RPC (within 2 months) and after a week of undergoing restorative surgery where their bowel was back in continuity (ileostomy closed). Samples were repeated at six months and one year. Familial adenomatous polyposis patients were used as a control arm and followed the same sample collection timings.

11.3.2.1.2 *Study 2 design: treatment cohort*

Patients were originally reviewed in a specialised pouch clinic at our centre. During this visit PIP was confirmed using clinical history, physical examination, blood tests, pouchoscopy and MRI scan (if applicable). When PIP was confirmed patients were offered an antibiotic regimen (if not already on antibiotics) and reviewed again in 4-6 weeks following antibiotic treatment.

Patients with chronic PIP already on long-term antibiotics were considered for the study, and if they were clinically stable a withdrawal of antibiotics was encouraged for 4-6 weeks ,when they were reviewed again in clinic. Patients were given the safety net of restarting antibiotics should they have significant deterioration in symptoms.

Had a patient required antibiotic use within the last two weeks of clinical review they were analysed as having taken antibiotics.

Table 27. Antibiotics used in study.

Antibiotic	Class of antibiotic	Coverage	Dose	Mechanism of action
Ciprofloxacin	Fluroquinolone	Broad spectrum against gram positive and gram-negative bacteria	500mg BD	Inhibits DNA gyrase and type 2 topoismerase, topoismerase V
Co-amoxiclav	Penicillin	Broad spectrum against gram positive and negative	625mg BD	binds to penicillin-binding proteins within the bacterial cell wall and inhibits bacterial cell wall synthesis. Clavulanic acid is a β-lactam, structurally related to penicillin, that may inactivate certain β-lactamase enzymes.
Metronidazole	Nitroimadazoles	Anaerobic gram positive and negative bacilli Anaerobic cocci Protozoa	400mg BD	Exerts action on susceptible organisms in four successive stages: entry of the drug into the organism, its reductive activation, interaction of the reduced intermediate products with intracellular targets, and breakdown of the toxic intermediate products.

11.3.2.2 Outcome definitions

PIP was defined using the pouch disease activity index (PDAI) [241]. A score of greater than 7 was considered as PIP. Chronic PIP was defined as those patients who required antibiotics for more than three occasions within the last year to control pouch

related symptoms. Treatment response was defined by a two or more-point reduction in the PDAI or an overall score of less than seven. PDAI was not recorded on the first time-point as it was deemed too close to surgery to enable an accurate reflection of inflammation.

11.3.2.3 Serum sample collection

6mL of blood in a sterile blood was collected in lithium heparin tubes (6mL) (BDbioscience). Samples were then immediately centrifuged at 1200*g* at 4°C for 20 min. The supernatant was then drawn off and placed in Eppendorf tubes stored at -80°C until further sample preparation. Samples were then fully thawed at room temperature.

11.3.2.4 Urine sample collection

A 113ml wide mouth, sterile, polypropylene container was used to collect urine. A mid-stream morning sample was collected from each patient. The urine was then transferred using a sterile transfer pipette. The volume transferred was between 1mL and 1.2mL into three 1.5mL screwcap tubes.

All samples were stored for no greater than six months.

11.3.2.5 NMR buffer preparation for serum

NMR buffer was prepared by dissolving 10.05g $Na_2HPO_4x7H_2O$ in 380mL H_2O, 0.4g of TSP (0.08 % w/v, 3-trimethylsilyl-[2,2,3,3,-$^2H_4$]-propionic acid sodium salt) as an internal chemical shift reference was then added. This was then mixed with 5ml of a 4% (w/w) NaN_3/H_2O-solution. The resulting solution was then adjusted to pH 7.4 using 1M HCl (1M NaOH). The buffer was then filled it up to 400mL with H_2O, 100mL of D_2O was added and mixed well. This was then stored at 4°C until further analysis with previous studies highlighting stability[377]. Standard protocol for 100 mL buffer was then applied: 80mL of H_2O, 20mL of D_2O, 2.01g of sodium phosphate dibasic heptahydrate, 80mg of TSP, and 1 mL of 4% (w/w) sodium azide solution were used. This was then mixed until the chemicals had dissolved. The pH was then adjusted to 7.4 with 1M HCl (1M NaOH). The buffer was then stored at 4 °C.

11.3.2.6 NMR buffer preparation for urine

Standard Bruker protocol was used: To make the buffer we dissolved 10.2g KH_2PO_4 in 40mL D_2O and dissolved 50mg TSP and 6.5 mg of NaN_3 in 3-5mL D_2O. This was then mixed very well. The pH was then adjusted to 7.4 with 1M HCl. This was then filled up to 50mL with D_2O and mixed very well. This was stored at 4°C until further analysis. To make 100mL of buffer 100ml D_2O was poured into a volumetric flask. Following this 2.84g of Na_2HPO_4, 0.48g NaH_2PO_4, 17.2mg TSP and 19.5mg NaN_3/Mix were mixed until chemicals have dissolved. pH was adjusted to 7.4 using concentrated HCl. This was stored at 4°C until further analysis.

11.3.2.7 Sample preparation of serum

300μL of serum was added to 300μL of buffer in Eppendorf tubes. Tubes were then centrifuged at 12,000g for 5min at 4°C, and 575μL of sample was transferred into 5mm NMR tubes.

11.3.2.8 Sample preparation for urine

Samples were thoroughly thawed at room temperature, each sample was vortexed for 5s, samples were then centrifuged for 10 min at 10.621g and 4°C. Following this 1.5mL Eppendorf tubes were placed on a rack where 60μL of Human Urine Buffer (pH 7.4, 100% D_2O) and 540 μL of urine supernatant were placed into each tube. This was then vortexed and was allowed to stand for 5 min. 575μL of supernatant was then transferred into a 5mm NMR tubes.

11.3.2.9 NMR Spectroscopy parameters

The spectra were acquired on a Bruker® 600 MHz Avance III spectrometer, with a Samplejet 96 well autosampler. Standard 1D ^1H NMR experiments with water suppression (called in Bruker system: noesygppr1d) was performed at 300 K for urine and 310 K for serum using the following parameters: Relaxation delay, 4s; mixing time,10ms acquisition time, 2.726s and spectral width, 20 ppm. For serum samples, following the acquisition of the 1D NOESY-presat, 1D CPMG with water saturation was acquired using the Carr–Purcell–Meiboom–Gill pulse sequence with a spin-echo delay of 0.3ms and the implementation of 128 loops for T_2 filter. The resulting free induction decays (FID) were Fourier transformed, then a line-broadening factor of 0.3

Hz and zero filling factor of 2 was applied producing NMR spectra with 132K data points. All NMR spectra were automatically referenced to TSP at 0 ppm and to the anomeric proton of α-glucose at 5.23 ppm for urine and serum, respectively. The spectra were also automatically phased and baseline-corrected on Topspin 3.2 (Biospin).

11.3.2.10 NMR pre-processing

Full resolution NMR spectra were exported to Matlab (Matlab R2014a) for pre-processing. Spectral regions corresponding to the internal standard (δ -0.5 to 0.2), water (δ 4.5 to 4.8), and noise (δ 9.8 to 10) were excluded from the analysis. The spectra were not aligned.

11.3.2.11 Multivariate data analysis

The full resolution 1D ^{1}H NMR spectra were imported into the SIMCA-P software package (v14.1, Umetrics, Sweden) and multivariate data analyses were carried out. Initially, the principal component analysis (PCA) of the NMR data set was performed (on mean-centred data and unit variance scale) to visualize the stability of the run and to identify outliers (based on the principles of Hotelling T^2) within the data set. The supervised multivariate methods, using Partial Least Squares with Discriminant Analysis (PLS-DA) and Orthogonal-Projection to Latent Structure with Discriminant Analysis (OPLS-DA) was performed. This procedure results in a cross-validation parameter Q^2Y, indicating the predictability of the model. The R^2Y indicates the amount of variance in Y (the outcome) explained by the model. The values of R^2Y and Q^2Y are used to evaluate possibly over-fitted models. A final significance test was performed with the use of a CV-ANOVA (analysis of variance of the cross-validated residuals) to verify the models' validity.

11.3.2.12 Markers identification and assignment

Once the NMR spectral regions related to the discrimination between two sample classes had been identified using supervised multivariate discriminant analysis, Statistical Total Correlation Spectroscopy (STOCSY) as well as SubseT Optimization by Reference Matching (STORM)[360] was applied to help metabolite assignment. STOCSY takes advantage of the multicolinearity of the intensity variables in a set of

spectra to display the correlation among the intensities of the various peaks across the whole sample. Added information is available by examining lower correlation coefficients or even negative correlations, since this leads to the connection between two or more molecular species involved in the same biochemical process. Metabolite assignment is also performed by comparing chemical shifts, multiplicity, and peaks intensity ratio with information reported in databases such as the Human Metabolome Data Base[358] or the Biological Magnetic Resonance Data Bank[378] and previously published literature[379][380]

11.3.3 Results Study 1 – Longitudinal assessment of pouch over a year

11.3.3.1 Baseline characteristics

There were 20 patients who were followed-up longitudinally. These comprised six patients with FAP and 14 with UC. The total number of samples analysed was 39, including quality controls. There were 21 samples available taken at closure of ileostomy and 14 samples taken at either 6 months or 12 months following closure of ileostomy. The median age of the patients at baseline was 37 (range 20-62). There were 11 males and 9 females. I assessed if gender contributed to differences in spectra and therefore could be a potential confounding factor.

Seven of the UC patients developed PIP within a year of follow up based on the PDAI.

Table 28. Sample point collection and corresponding PDAI scores. Where a sample was collected and analysed it was marked with an “x”.

	Patient ID	Time point 1	Time point 2		Time point 3	
		Samples collected	Samples collected	PDAI score	Samples collected	PDAI score
FAP patients	**FAP 1**	x	x	<7		
	FAP 2	x	x	<7	x	<7
	FAP 3	x	x	<7		
	FAP 4	x				
	FAP 5	x				
	FAP 6	x			x	<7
IBD patients	**UC 1**	x				
	UC 2	x				

Patient ID	Time point 1	Time point 2		Time point 3	
	Samples collected	Samples collected	PDAI score	Samples collected	PDAI score
UC 3	x	x	<7	x	8
UC 4	x			x	8
UC 5	x			x	9
UC 6	x	x		x	8
UC 7	x	x	<7	x	10
UC 8	x				
UC 9	x	x	<7		
UC 10	x	x	<7		
UC 11	x				
UC 12	x	x	<7		
UC 13	x	x	<7	x	8
UC 14	x	x	<7	x	8

There were a variety of comparisons made in the longitudinal samples (see appendix 2). There were no significant metabolic differences between those that developed PIP and those that did not develop PIP at any of the time points assessed. There were no significant metabolic differences between UC patients at different timepoints and no significant metabolic differences between FAP patients at different time points. Furthermore, there were no significant differences between the UC patients and FAP patients across any of the time points assessed.

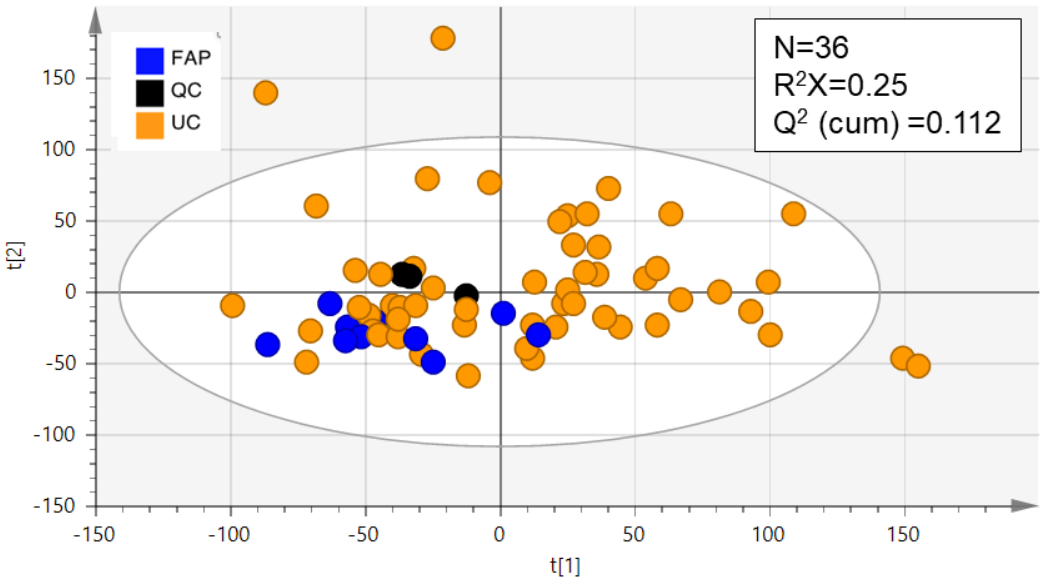


Figure 34. PCA scores plot of all urine samples (UV scaling) showing the robustness of the run (clustered QCs).

When we considered all male patients and all female patients longitudinally together there were significant differences detected between males and females. This model is further explored below

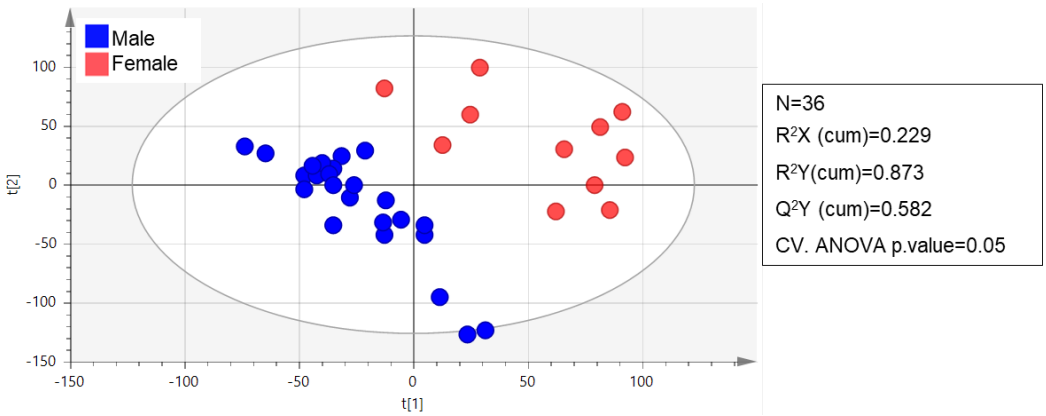


Figure 35. PLS plot showing differences in males vs female UV scaling.

Table 29. values of significant model for urine longitudinal.

Comparison	All longitudinal samples	Male *vs* Female	Male *vs* Female
Model type	PCA	PLS	PLS
Scaling	UV	UV	PARETO
Components	2	2	2
Number of samples included	36	36	36
R^2X (cum)	0.25	0.229	0.261
R^2Y(cum)		0.873	0.814
Q^2(cum)	0.112	0.582	0.546
***P*-value**		$3.33x10^{-5}$	$5.19x10^{-5}$

All models were compared using UV and Pareto scaling. The full comparisons can be found in (appendix 2). The best model (Pareto scaling) was taken forward for analysis. *P*-values are from CV-ANOVA testing

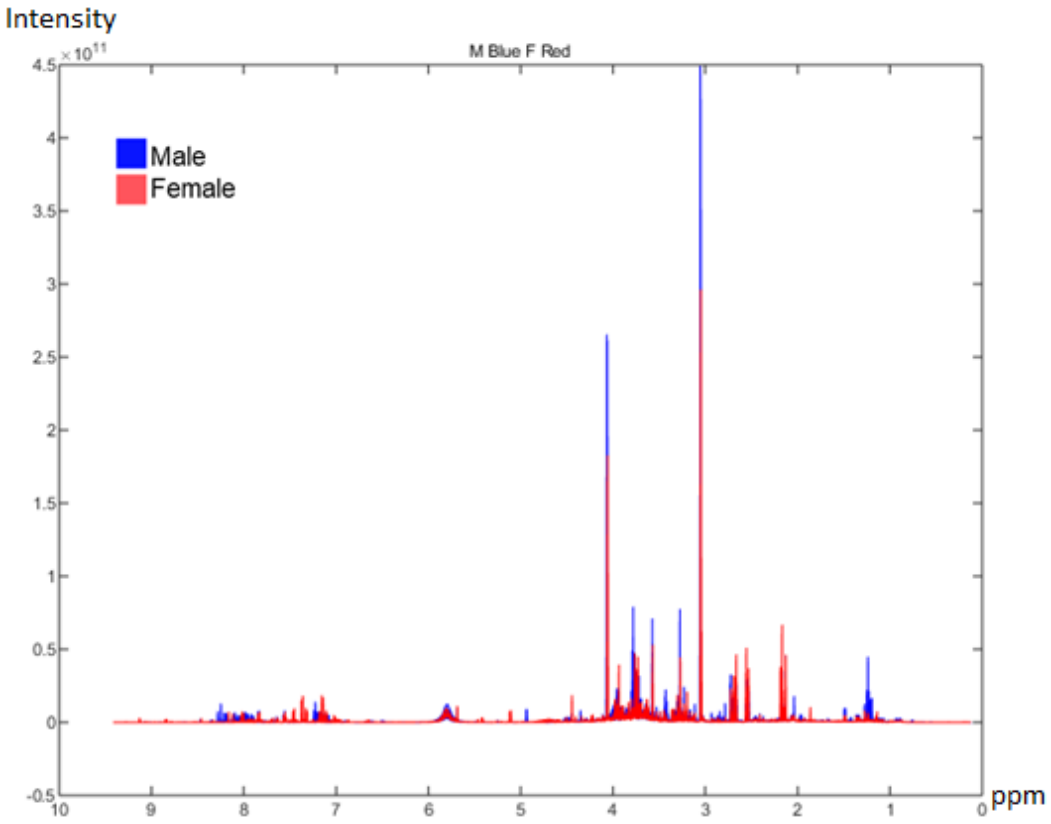


Figure 36. Spectra associated with significant changes Male vs Female.

The above model highlights the average spectra of all urine samples that were included in the model for gender.

Table 30. Male vs female significant peaks identified.

PPM (multiplicity)	Relations through STORM	Increase observed	Identification
1.49(d)	0.9881,1.04,1.092, 1.102,1.112,1.34, 1.361,1.48, 1.492,1.925, 2.032, 2.237,2.368,2.465, 2.649, 3.569,3.953, 5.242, 5.629,5.636,5.694,5.697, 6.047,6.049, 6.091,6.098, 6.108, 7.558, 7.571, 7.645,7.657,7.848, 9.143,9.151, 9.17,	M>F	Alanine
3.05(s)/4.06(s)	No correlations	M>F	Creatinine
1.365(s)		M>F	2-Hydroxyisobutyrate
2.93(s)	2.238,2.722,3.142,4.308	M>F	Dimethylglycine

2-Furoylglycine (females), Ethylglucuronide (males) were found to be increased but these were considered related to coffee and alcohol consumption respectively[381]. In particular N-acetyl-S-(1Z)-propenyl-cysteine-sulfoxide (NAcSPCSO) and its isomer were also found, which is the biomarker of onion consumption[381].

11.3.4 Results study 2 treatment arm (on and off antibiotics)

There were 22 patients of which 5 were heathy controls who were not on antibiotics. One sample was lost in the processing of the sample, making 26 samples available for analysis. The median age of the cohort was 50 years old (28-79). The median length of time from closure of ileostomy to sample collection was 8 years (range 1-29 years). There were 9 females and 18 males in the cohort.

There was a total of 12 patients on antibiotics and 10 patients off antibiotics. All samples were taken in the morning. Five patients were using ciprofloxacin and metronidazole in combination, four were using ciprofloxacin only, two were using co-amoxiclav only and one was using a combination of trimethoprim and ciprofloxacin. For patients to be considered off antibiotics, patients were required to be off them for at least two weeks.

11.3.4.1 Serum

There were 30 samples in the cohort consisting of 20 patients, 3 QCs. Six patients had paired on and off antibiotic samples. There were 5 healthy controls. The median age of the cohort was 49 (range 19-61). There were 12 samples taken on antibiotics and 10 samples taken off antibiotics. There were 12 males and eight females. Antibiotics used were ciprofloxacin and metronidazole in four patients, co-amoxiclav and ciprofloxacin in three patients, ciprofloxacin in two patients, co-amoxiclav in two patients, metronidazole in one patient and trimethoprim and co-amoxiclav in one patient.

11.3.4.2 Results from models

11.3.4.2.1 *Urine*

When comparison was made between responders on vs off antibiotics and non-responders on vs off antibiotics there were no significant differences found. There were not enough observations to compare responders vs non-responders off antibiotics. We therefore looked at responder' vs non-responders overall. There were differences detected on NMR between responders vs non-responders.

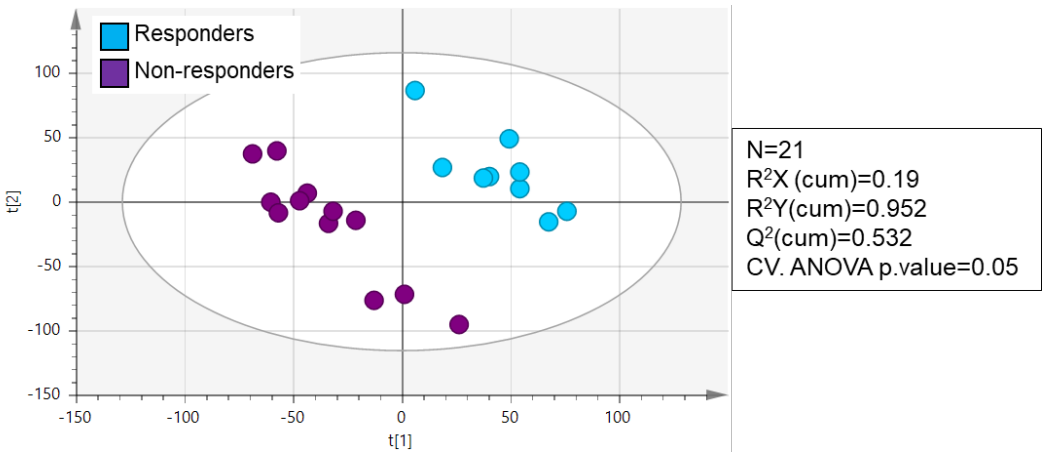


Figure 37. Model 6 UV PLS-DA showing differences between responders vs non-responders.

Table 31. Significant differences detected Responder vs Non-Responders and multiplicities.

	Compounds	ppm
Non-responder *vs.* Responder	Formate	8.84(s)
	Trigonelline/1-Methylnicotinamide	4.45(s), 8.85(m), 9.13(s)
	Glycine	3.57(s)

There were significant increases in formate, trigonelline/1-methylnicotinamide and glycine in patients who were responders to treatment vs non-responders.

11.3.4.2.2 *Serum*

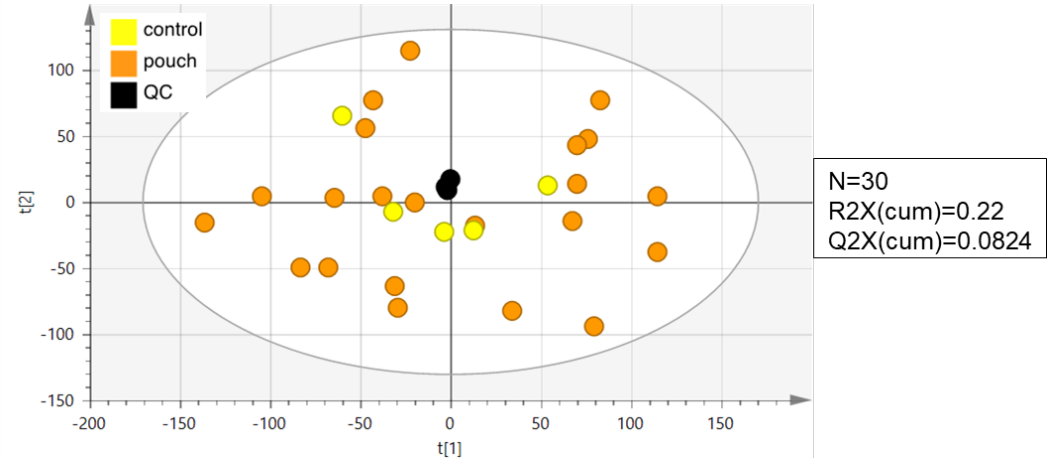


Figure 38. A PCA-X plot highlighting a stable run with the QC's all relatively central.

There were no differences using supervised and unsupervised models between the responders vs non-responders and between those on and off antibiotics (table 32).

*There were not enough observations and patient numbers to compare those off antibiotics responders vs non responders and therefore will affect the performance of the statistical models.

Table 32. Responders vs non-responders.

Comparison	Model type	Scaling	Components	Number of samples included	R^2X (cum)	R^2Y(cum)	Q^2(cum)	*P*-value
ALL samples	PCA	UV	2	30	0.22		0.0824	
All samples without QC	PCA	UV	2	27	0.23		0.0729	0.79
Responder *vs*. non-responder	OPLS-DA	UV	1+1	22	0.199	0.963	0.0904	0.87
	OPLS-DA	PAR		22	0.289	0.545	-0.265	1
	OPLS-DA	UV	1+1	18	0.218	0.979	0.084	1
	OPLS-DA	PAR	1+1	18	0.637	0.61	-0.553	1
On *vs.* off abx	OPLS-DA	UV	1+1	22	0.193	0.955	0.0852	0.808802
On vs off abx	OPLS-DA	PAR	1+1	22	0.659	0.362	-0.296	1
On abx responders vs on non-responders	OPLS-DA	UV	1+1	15	0.212	0.986	0.0592	0.96
On abx responders vs On non-responders	OPLS-DA	PAR	1+1	15	0.471	0.688	-0.355	1

11.3.5 Discussion

11.3.5.1 Study 1 longitudinal

My data have highlighted that there are metabolic differences in my longitudinal cohort between males and females this is a well-documented phenomenon in NMR[382,383]. Significantly we found that males had significantly higher proportions of alanine, creatinine, 2-hydroxyisobutyrate and dimethylglycine.

The likely reason creatinine is higher in males is due to muscle mass. It is well established that creatinine is a breakdown product of creatine phosphate in muscle. As males usually have a higher muscle mass, this is likely to account for this difference observed.

Alanine is a non-essential amino acid and in terms of IBD has been suggested to be increased in IBD patients when compared to healthy controls[384]. Interestingly alanine again is found in high concentrations in the muscles so may also account for these gender differences. Alanine is essential amino acid for lymphocyte regulation[385] and requires the enzymes that are dependent on vitamin B6. As shown when comparing my responders to non-responders, the B vitamins may be associated with inflammation. Whether this difference observed has any role in pathogenesesis of inflammation in males and females however is yet to be established.

2-Hydroxyisobutyrate is primarily a breakdown product of liver tissue that can catabolize L-threonine or synthesise glutathione[386]. It has been established that in times of oxidative stress 2-hydroxyisobutyrate production tends to increase the production of glutathione. It is unclear why these differences between men and women were observed.

Dimethylglycine is a derivative of the amino acid glycine and can be found in beans and liver. It is also synthesized in the Krebs cycle. It is also found in high concentrations in legumes and is a dietary biomarker of it. It is therefore difficult to draw conclusions about the differences observed.

Despite these findings, there were no significant metabolic differences detected longitudinally between both UC patients and FAP patients. Therefore, in terms of the longitudinal analysis the H_a hypothesis are rejected.

11.3.5.2 Study 2 treatment cohort

My data have shown that there may be certain metabolites that are different between those patients that responders to treatment and those that do not respond to treatment. In particular, non-responders have higher levels of trigonelline/1-methylnicotinamide, formate and glycine.

Trigonelline is an alkaloid which is formed by the methylation of a nitrogen atom of niacin (vitamin B_3) and is a by-product of its metabolism and is excreted in human urine[387]. Methylnicotinamide is also a metabolite of niacin. In a mouse model of IBD (IL-10 deficient mice) this metabolite was shown to be important in females with IBD[388].Interestingly a study in 2004 found that Trigonelline was decreased in

patients with active IBD compared to those without active IBD. The same was found in a study which showed that IBD patients have less Trigonelline than healthy controls in the urine using NMR[389].

Formate is an essential metabolite in virtually all living organisms. The same study above suggested that formate differentiated healthy patients from IBD[389]. A further study supported this and found a lower levels of formate in inflamed intestines and suggested that formate is required locally to inflamed tissues in high volumes and therefore may account for a lower volume in urine.[390] Furthermore, Williams et al highlighted that formate levels were lower in patients with CD compared with UC[372]. Interestingly Formate is a short chain fatty acid, suggesting that high levels of formate is important in maintenance of health.

Glycine can be synthesized from serine but is an essential amino acid that appears to reduce oxidative stress[391].Glycine supplementation has been shown in animal models of IBD to reduce diarrhoea, body weight loss and ulceration in rats [392]. It has also been shown that glycine can downregulate proinflammatory cytokines and can enhance protein mass via regulation of TLR4, NOD, AMPK and mTOR signalling[393]. It is therefore unclear why this may be increased in non-responders but may reflect the need for glycine to be utilised locally in inflamed tissues but the role glycine in IBD is yet to be fully defined.

In terms of my hypothesis ,we were unable to detect significant differences between those on antibiotics compared with those not on antibiotics. In terms of my hypothesis in the treatment cohort I reject the H_a hypothesis.

11.3.6 Discussion summary

There is a paucity of data on the metabolic profile in PIP using NMR. Von Roon *et al* found increased levels of alpha-1 acid glycoprotein in human plasma[394]. Preter *et al* found that butyrate oxidation was reduced in patients with PIP compared with healthy controls but this was not measured through NMR techniques[395]. My data are therefore novel in this field and may help us understand the metabolic changes involved in PIP.

My data have several limitations. Importantly this study was not controlled for diet. Tigonelline could be a marker of caffeine and soya bean ingestion and therefore this could be an important confounder. It is possible that diet may influence some of the metabolites produced but this is very difficult to control for. It is also important to note that patients were not matched for co-morbidities and the use of other medications which may have had an effect on the results. The study is also limited by small number of patients included.

A significant weakness in the studies is that the patients were very heterogenous and my sample size did not allow for direct comparison between the same patient on and off antibiotics. Responders and non-responders were grouped together independent of the treatment they received as there were no significant findings when these were separated out between those on antibiotics and those off antibiotics. This means that results need to be interpreted with caution.

Future work should be repeated on bigger numbers. Future work should account for diet and attempt to match patients based on co-morbidities, drugs and diet.

11.3.7 Conclusion

Trigonelline/1-Methylnicotinamide, formate and glycine may help differentiate patients with PIP who will respond to treatments versus those that do not. It is currently unclear as to why these metabolites are reduced in non-responders and further work is needed to discover their potential role in response of PIP to treatment.

Chapter 12

Mass spectrometry Introduction

Mass spectrometry is an analytical process that ionizes chemical substances and separates them based on their mass-to-charge-ratio. The mass spectrum produces a plot of an ion signal in relation to its mass-to-charge-ratio. Spectra are presented in terms of Daltons per unit charge.

There are three main parts to a mass spectrometer: an ionization source, a mass analyser and a detector.

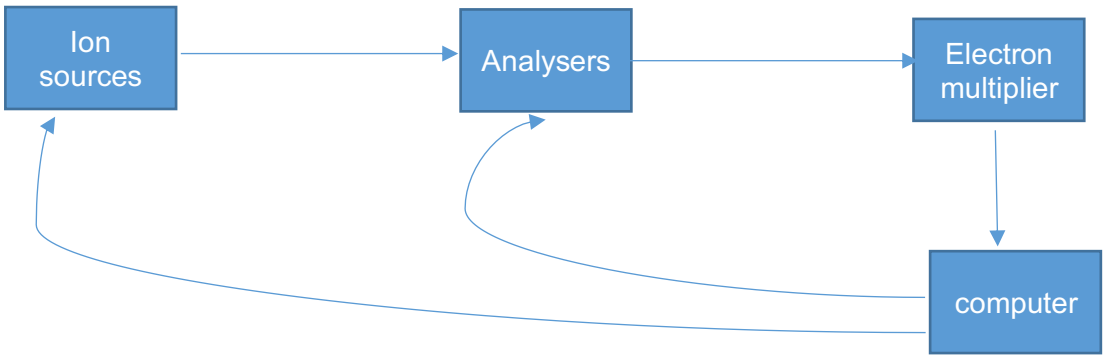


Figure 39. Parts of a mass spectrometer.

12.1 Mass spectrometry process

12.1.1 Ionization

The first stage of mass spectrometry is the conversion of the biofluid of interest into gas phase ions. There are many methods that ionize the biofluid with the most common being electron spray ionization[396] (ESI), and matrix-assisted laser desorption[397].

12.1.2 Electrospray ionization

In this method ions are generated at atmospheric pressure. A solution-based samples is passed through a small capillary that is at a potential difference relative to a counter

electrode at voltages between +500 and 4500 volts[398]. This high voltage that is applied to the liquid forms an aerosol. Sometimes a gas such as nitrogen is used to help this process. This then creates charged droplets, with a net positive or negative charge. Ions then become free from the solvent and make their way to the vacuum region of the mass spectrometer.

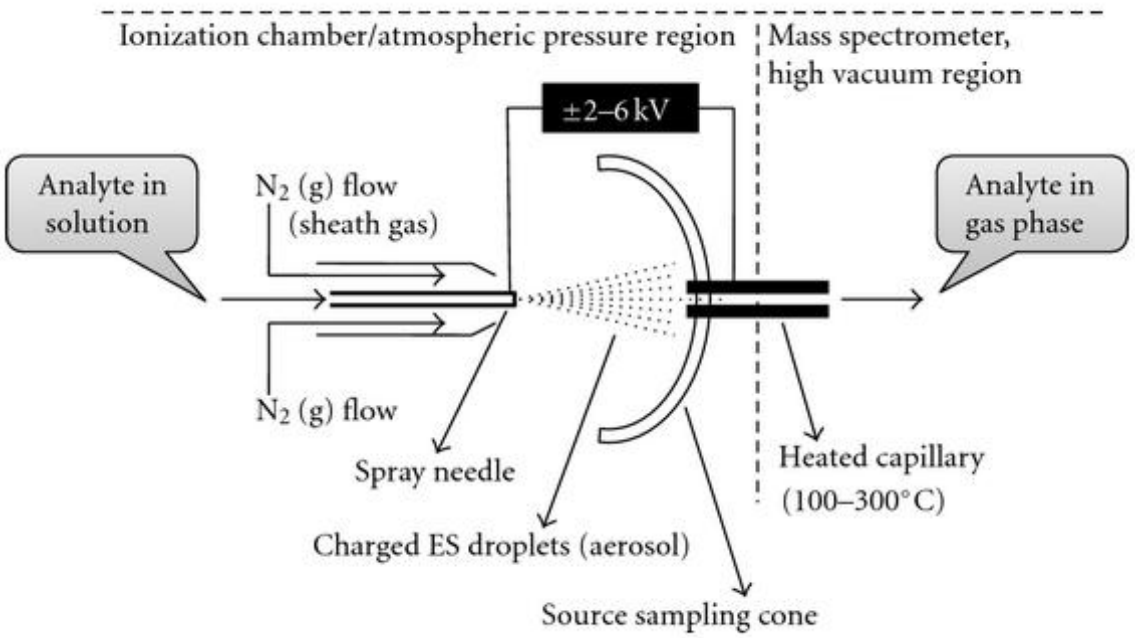


Figure 40. Process of mass spectrometer.

Reproduced with permission from [399].

12.1.3 Mass analysers

There are two types of mass analysers split into beam analysers or trapping analysers. In beam analysers the ion source pass through the analysing field detector in a beam. In trapped analysis the ions are trapped within an analysing field before being taken up by the analyser itself[398]

12.1.4 Detectors

The detectors record the charge induced or the current produced when an ion passes by or hits the detector. In many situations an amplifier is used to amplify the signal.

Gas Chromatography- Mass Spectrometry (GCMS)

12.1.5 Gas chromatography (GC)

Gas chromatography (GC) is an analytic method that allows separation and analysis of compounds that can be vapourised without degradation. GC- Mass spectrometry combines the techniques of gas chromatography with mass spectrometry.

12.2 Instrumentation

The GC-MS machine has essentially two components, one the gas chromatograph and the other the mass spectrometer (detailed above)

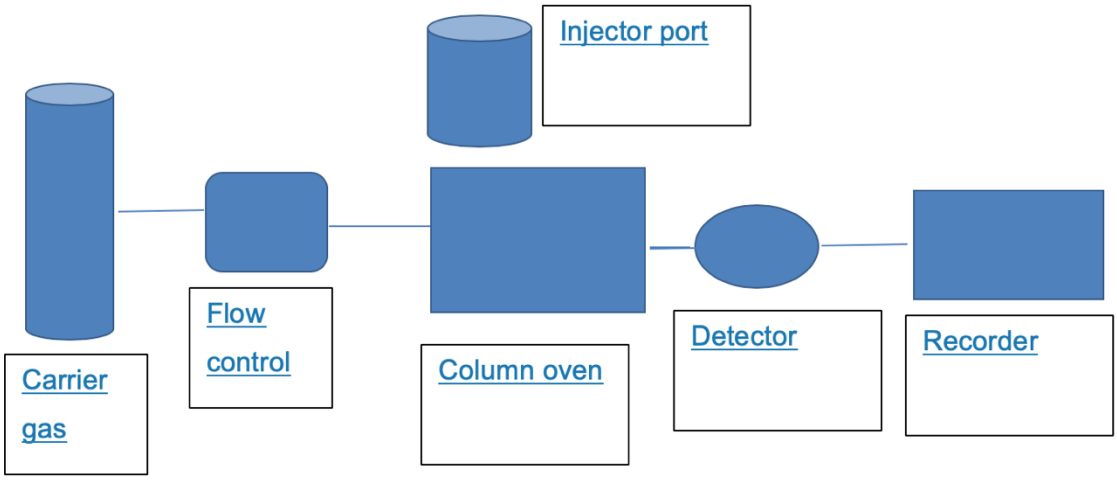


Figure 41. Schematic of a GC-MS machine.

12.2.1 Carrier gas

This is an inert gas which can include nitrogen, helium, argon and carbon dioxide. The carrier gas column also removes water and other impurities via a sieve-like structure.

12.2.2 Injector port

This is where the sample is injected into the column. Often a microsyringe is used as an injector into the column.

12.2.3 Column oven

This is a heated oven which controls the temperature of the column within tenths of a degree[400].

12.2.4 Column

This is a narrow tube which is coated with the stationary phase on its interior surface. The different compounds interact with the stationary phase on the column. It culminates in the most volatile substances leaving the column first. Columns differ in size, diameter and material dependent on the type of biofluid analysed.

Below is the gilent 7890B GC system, coupled to an Agilent 5977A mass detector (Agilent, Santa Clara) that was used for analysis

Figure 42. Agilent 59771 Mass detector.

12.3 Gas Chromatography Mass Spectrometry in Restorative Proctocolectomy

Short chain fatty acids (SCFA) are organic fatty acids with 1-6 carbons which arise from bacterial metabolism from `malabsorbed' carbohydrates entering the colon along with hydrogen gas[401]. They are final products of fermentation of dietary fibres by the anaerobic intestinal microbiota. They are the most abundant products derived from the commensal gut microbiota fermentation of indigestible dietary fibres[402]. The most common SCFA in the human gut are acetic acid, propionic acid and butyric acid which constitute over 95% of all the SCFA[403]. Humans lack the enzymes required to break down the bulk of dietary fibre, meaning that most passes into the large intestine unaffected. The fermentation of the this dietary fibre results in many metabolic substances being produced of which SCFA are the most common[404]. The concentration of SCFA varies across the length of the colon but ranges from 70 to 140mM in the proximal colon to 20-70mM in the distal colon[405]. The production of SCFAs relies on an interplay between the diet and specific diversity of the gut microbiota[406].Resistant starches make up 10-20% of all starch in the Western diet[407], furthermore sugars such as lactose, raffinose and stachyose may not be absorbed in the small intestine and may enter the colon where they are fermented. Therefore diets high in fibre, resistant starches and complex carbohydrates will lead to an increased rate of SCFA formation[401]. Dietary intake of fibre or resistant starch are linked to enhanced SCFAs production in gut as well as in peripheral blood[404,408].

Of particular interest in relation to inflammatory bowel disease are the SCFAs acetic acid, propionic acid, and butyric acid, which are solely metabolized by gut bacteria from otherwise indigestible carbohydrates[405].These have been particularly of interest in animal models where it has shown that high levels of these SFCA can ameliorate colitis[409]. Another SCFA of interest is lactic acid. This is an intermediary product of carbohydrate fermentation and accumulates only when SCFA production is inhibited in an acidic milieu of pH less than 5.5[407].

Specifically in IBD, a decrease in SCFA in particular butyric acid has been identified as being associated with dysbiosis, which in itself has been associated with inflammation in IBD[410].

12.3.1 Metabolism of SCFA

There are four main proposed mechanisms for absorption of SCFA into colonocytes., including non-ionic diffusion[411], exchange with bicarbonate on a 1:1 ratio[412,413], co-transportation with cations via the hydrogen-coupled monocarboxylate transporters (MCT1, MCT2, and MCT4)[414] and co-transportation via sodium coupled monocarboxylate transporter 1 (SMCT1)[415]

SCFAs that pass into the colon are absorbed by colonocytes where they can be used locally for fuel for the colonic mucosal epithelial cells[401]. Butyric acid is the most important SCFA in this process and accounts for 70-90% of metabolism by the colonocyte[401]. Butyric acid is used preferentially over propionic acid and acetic acid at a ratio 90:30:50[401]. It has been estimated that less than 10% of the SCFAs are excreted in faeces[416].

12.3.2 Specific SCFA producing microbiota

Acetic acid has been shown to be produced from pyruvate via two different mechanisms. One via the acetyl-CoA pathway by the enteric bacteria and the other via Wood-Ljungdahl pathway by acetogens such as *Blautia hydrogenotrophica*[405]. Butyric acid has been shown to be produced from Acetyl-CoA by several of the Firmicutes family. Propionic acid is also produced by two different pathways including the succinate pathway by Bacteroidetes and the lactic acid pathway by Firmicutes[417]. Through next generation sequencing technologies, we have been able to begin to isolate specific bacterial taxa that are associated with SCFA. Propionic acid seems to be produced from a variety of bacterial groups whereas propionic acid production appears to be dominated by a few specific organisms with some of the deoxy-sugars such as fructose and rhamose aiding the production of propionic acid in select bacterial groups[418]. In terms of Butyric acid production it has been shown that *Faecalibacterium prausnitzii, Eubacterium rectale, Eubacterium hallii* and *Ruminococcus bromii*, are responsible for the production of the majority of butyric acids[419]. Furthermore, it has been highlighted that resistant starches significantly contribute to butyric acid production in the colon which is dominated by *Ruminococcus bromii*[420].

12.3.3 SCFA and IBD

Various studies have implicated the SCFAs to have a role in both the development of IBD and flares of IBD. It has been shown that there is a decrease in total faecal SCFA in children from Europe when compared with those from Burkina Faso. As children from Africa rarely get IBD it has been suggested that SCFAs may therefore play a role in the development of IBD[421]. Suggested reasons for differences found in SCFAs in this study included the differing diets. Validating these findings was an animal study which highlighted that a Western diet caused microbiome perturbation, SCFA reduction and high risk of colitis[422]. Translating this into a human model, it has been shown that total SCFAs are significantly lower in those with IBD when compared with healthy controls[423]. This has been further supported by a recent study which highlighted that the butyric acid producing species *Roseburia hominis* and *Eaecalibacterium prausnitizi* were reduced in those patients with UC[424].

12.3.4 SCFA in the pouch

It has been suggested that SCFAs increase as the pouch adapts. Hove *et al* highlighted that the concentrations of faecal SCFAs were low in non-adapted pouches (mean +/- SE, 20.3 +/- 3.4 mmol/l) which increased to intermediate levels, 53.3 +/- 8.4 mmol/l, between 6 months and a year after ileostomy closure, and 96.3 +/- 7.9 mmol/l, after more than a year of adaptation[425].

Historically there has been interest in the role of SCFAs in PIP. With several reports highlighting that decrease in SCFAs may be associated with PIP and can be used as a therapy to successfully treat PIP[426]. In one such study it was shown that faecal samples in patients with PIP had a mean SCFA concentration of 17.5 mmol/l ± 3.4 compared with 33.3 mmol/l ± 5.5 (p value <0.05) in healthy controls. However this has not been validated in other studies which have suggested that SCFAs show no differences between healthy controls and those with a PIP[427].

12.3.5 SCFAs as treatment of PIP

SCFAs have been used as a potential therapeutic treatment. Fibre as a source of SCFAs was trialled in a group of 13 patients in a nine-week cross over study , who were given either pectin or methyl cellulose for a 2-week period. This trial suggested that increase intake of these fibres had no effect on stool frequency or pouch

function[428]. A further study explored the role of inulin which is fermented to SCFAs. In this randomised placebo-controlled trial, patients in the treatment arm received 24g/day of inulin. The patients that received inulin demonstrated increases in butyric acid concentrations (18.9 mmol/g vs. 11.7 mmol/g $p < 0.01$) compared with patients on placebo. In the Inulin treated group the overall PDAI score was lower (4.05 vs. 5.39, $p< 0.01$) with significantly lower endoscopic (0.95 vs. 1.47, $p < 0.04$) and histologic scores (2.11 vs. 2.61, $p <0.04$) when compared with placebo[429]. The key weakness of this study is that none of the patients enrolled had PIP meaning that the value of inulin remains unclear.

Den Hoed *et al* demonstrated that SCFAs enema containing sodium acetate (60 mmol/L), sodium propionate (30 mmol/L) and sodium *n*- butyrate (40 mmol/L) made in isotonic solution by the addition of sodium chloride given twice a day for 4 weeks could completely cure PIP in a 48-year old lady[430]. Furthermore, there have been some short series exploring the use of SCFAs in the form of enemas. Two studies explored the use of a SCFAs enema with the formulation of 60 mmol/L sodium acetate, 30 mmol/L sodium propionate, and 40 mmol/L sodium *n*-butyrate in a combined total of 10 patients, all of whom had chronic PIP[431,432]. Following these enemas, only 2 of the patients had a clinical response and two patients had worsening symptoms.

To date there has been one study which randomised patients with PIP to receive either butyric acid suppositories or glutamic acid suppositories for 21 days. The results highlighted similar response in both arms with no recurrence of symptoms in six of the 10 and three of the 9 patients who received glutamic acid suppositories butyric acid suppositories respectively. They concluded that more studies were needed on the effect of SCFA in the treatment of PIP[433].

12.4 **Hypothesis**

1. SCFAs concentrations will differ between responders vs non-responders
2. SCFAs concentrations will differ between those that develop PIP and those who do not, and may therefore predict those who may develop PIP

12.5 Methods

12.5.1 Patient recruitment

For both studies, patients above the age of 16 who were being considered for restorative proctocolectomy (RPC) were considered for the study. Patients were excluded if they were unable to provide written consent, were pregnant at the time of consent.

For both studies ethical approval was granted by the Brent Research Ethics Committee **ID:08/H0717/24: Prospective study of immunological and microbiological factors in inflammatory bowel disease.**

12.5.2 Study 1 design longitudinal cohort

Patients were recruited using a prospectively maintained pouch database at a single institution which highlighted potential candidates for restorative proctocolectomy. Patients who agreed to undertake the study were then reviewed where consent was obtained.

Patients provided an early morning mid-stream urine both prior to undergoing RPC (within 2 months), after a week of undergoing restorative surgery where their bowel was back in continuity (ileostomy closed). Samples were collected at 6 months and one-year post completion of restoration of continuity. At the same time intervals, biopsies were taken from the pouch body. Familial adenomatous polyposis patients were used as a control arm and followed the same sample collection timings.

12.5.3 Study 2 design treatment cohort

Patients were originally reviewed in a specialised pouch clinic. During this visit PIP was confirmed using clinical history, physical examination, blood tests, pouchoscopy and MRI scan (if applicable). When PIP was confirmed patients were offered an antibiotic regimen (if not already on antibiotics) and reviewed again in 4-6 weeks following antibiotic treatment. Urine, stool and serum were collected from patients with defined PIP both prior and after antibiotic treatment. Antibiotics were provided for a minimum of 2-4 weeks.

Patients with chronic PIP already on long-term antibiotics were considered for the study, if they were considered clinically stable a withdrawal of antibiotics was encouraged for 4-6 weeks where there were reviewed again in clinic. Patients were given the safety net of restarting antibiotics should they have significant deterioration in symptoms. Had a patient required antibiotic use within the last two weeks of clinical review they were analysed as having taken antibiotics.

12.6 Clinical data

The following clinical data points were collected: age, gender, past medical history, drug history including antibiotic use, age of pouch.

12.7 Outcome measures

12.7.1 Study 1

PIP was defined using the pouch disease activity index (PDAI)[241] and when the score was ≥ 7. The development of PIP was assessed at months 6 and 12 of the longitudinal study.

12.7.2 Study 2

PIP was defined as before. Response to antibiotics was defined as either a 2 points reduction in a patients PDAI or a score of <7. Patients were classified as off-antibiotics if they had stopped all antibiotics for a period of at least 2 weeks prior to sample collection.

12.8 Methods for collection

12.8.1 Stool

Stool samples were obtained at the time of consent. These were then aliquoted using sterile pipettes into Eppendorf's tubes and stored at -80°C in freezer until further GC-MS/MS analysis.

Samples were thawed to room temperature. MTBE (methyl *tert*-butyl ether) was added to methyl stearate internal standard (IS) and was stored at 4°C The defrosted stool was then dispensed into 2ml Eppendorf tube with a total weight of 50mg of stool per sample. This was then stored at 4°C. 500µL of MTBE with 500µL of internal standard

and 4µL of hydrochloric acid was then added. Each sample was then vortexed for 5 seconds. Samples were then shaken for 20 minutes at speed 5. Following this all samples were centrifuged at 10000 relative central force (rcf) for 5 minutes at 4°c.

Following this 30µL of the polar phase was placed into silanised vials followed by 150µL of derivatiser MTBSTF + 1% TBDMSCI (N-tert-Butyldimethylsilyl-N methyltrifluoroacetamide with 1% tert Butyldimethylchloro-silane). The cap of the vial was put on immediately after the derivatiser was added. These were then vortexed for 5 seconds and incubated in an oven for 45 min at 60°c. 70µL from the silanized vial was then taken and put into silanized inserts and placed into regular vials. For the quality controls (QCs) 15µL was taken from each sample and pooled into a falcon tube. This was then processed as described above for the regular samples.

12.8.2 Serum

Serum was taken at the time of the consent process. Whole blood was collected via venepuncture into 6mL sodium heparinized vacutainers. Tubes were then inverted ~10 times immediately after collection. Samples were centrifuged at 1600*g* for 15 min. The plasma supernatant was then transferred into a 5 mL Eppendorf tube and 0.5 mL was aliquoted in four different tubes and snap frozen to be then stored at -80°C until analysis.

Samples were thawed to room temperature and vortexed for 5 sec.

A volume of 100µL of samples was mixed to 250µL of MTBE and 250µL of IS was added to each sample. These were then vortexed and shaken for 20 minutes and then centrifuged at 9.5G for 5 min at 4°C.

90µL of the polar phase from each Eppendorf was placed into silanized 2ml amber vials. 60µL of derivatiser MTBSTF +1% Butyldimethylchloro-saline) was added and with the cap of the Eppendorf immediately applied. Samples were then incubated for 45min at 60°C in an oven. Following this 70µL of each sample was taken and placed in mass spec vials which were then placed in the silanized vials. For the QCs 15µL of each sample was taken and placed into silanized vials. In total there were 5 QCs.

12.8.3 Urine

Following an overnight fast, first morning urine was collected into sterile standard urine tube. Samples were immediately aliquoted and stored at -80°C until analysis.

12.8.4 Biopsies

Pouch mucosal biopsies were obtained during an endoscopic procedure using a standard single use biopsy forceps. Prior to undergoing pouch formation, terminal ileum samples were taken where possible. Two pouch biopsies were taken from the pouch body using the standard 2.2mm forceps (Boston Scientific, Marlborough, USA). Samples were then snap frozen immediately using liquid nitrogen and stored at -80°C until further analysis.

To prepare biopsies for GC-MS/MS analysis, samples were defrosted at room temperature. Samples were weighed, sterile water and MTBE+IS was added with a ratio of 20mg of sample:50µL of H_20:250µL of MTBE and IS. Following 4µL of hydrochloric acid was added to each sample.

Following completion of the solution beads were added to each Eppendorf. These then underwent bead beating for 5 secs. Each sample was then vortexed and centrifuged at 9.5 G for 5 min at 4°C.

30µL of the polar phase was then placed into silanized Eppendorf tubes. 150µL of derivatiser was added to each sample and the cap of the tube applied immediately. These were then incubated for 45 minutes at 60°C in an oven. 70µL from the silanised vial was placed into vial inserts and analysed in the GC-MS machine.

For QCs 20µL from the polar phase of each samples was taken and pooled in a falcon tube.

12.9 Statistical analysis

The quantification of SCFA were performed using an Agilent 7000C Triple Quadrupole GC/MS-MS System according to a previously published method[434]. We analysed 10 short chain fatty acids, 2-hydroxybutyric acid, lactic acid, isobutyric acid, acetic acid, propanoic acid, butyric acid, 2-methyl butyric acid, isovolaric acid, valeric acid and caproic acid.

Data processing was performed using the software Agilent MassHunter Quantitative Analysis software (Agilent, Santa Clara, California, USA) and SCFA concentrations were integrated from a calibration curve run at the start and at the end of the sample analysis.

Excel version 16 (Microsoft corporation, Redmond, Washington, USA) was used to calculate median and averages. Where data were normally distributed, 2-tailed t-test was used in excel to calculate differences across groupings. Data were adjusted for multiple analysis using the Bonferroni correction with an original statistical significance considered if $p<0.05$. Significance using the Bonferroni correction was therefore set at $p<0.01$

Simca version 14 (Umetrics, Malmo, Sweden) was used for multivariate analysis including principle component analysis (PCA), partial least square (PLS) and orthogonal partial least square-discriminant analysis (OPLS-DA).

Box whisker plots, networks and bar plots were generated using R (R Foundation for statistical computing, Vienna, Austria).

12.10 Results

12.10.1 Study 2 treatment cohort

There were 10 females and 18 males. The median age of the cohort was 47 (26-74). The median age of the patients with PIP was 47 (range 21-74). In total there were 6 patients who had paired pre- and post- antibiotic samples.

Table 33. faecal samples collected in treatment arm.

Type of patient	Number of patients	Samples on treatment	Samples off treatment
PIP	23	16	12
Healthy controls	5	n/a	5

12.10.1.1 Urine

The median age of the patients was 44 (range 21-65). There were 8 females and 12 males in the cohort. The median age of the pouch was 8 years (1-39 years) from closure of ileostomy. Four patients used ciprofloxacin and metronidazole in combination, two patients used co-amoxiclav, two patients used ciprofloxacin, one patient used Cefuroxime and one used trimethoprim.

Table 34. urine samples on and off antibiotics.

	n	Samples On antibiotics	Samples Off antibiotics
PIP	18	11	9
Healthy controls	5	n/a	5

12.10.1.2 Serum

Antibiotics were used in seven samples who were on ciprofloxacin and metronidazole, four were on ciprofloxacin and two were on Co-amoxiclav. The median age of the patients was 43 (21-64). There were ten females and 11 males. The median age of the patients with PIP was 47 (range 21-74). Six of these samples were paired samples (one patient gave three samples)

Table 35. Serum samples on and off antibiotics.

	n	Samples On antibiotics	Samples Off antibiotics
PIP	16	13	10
Healthy controls	5	n/a	5

Table 36. Summary table of biofluids.

	HC	on antibiotics	Off antibiotics
Faeces	5	16	12
Urine	5	11	9
Serum	5	13	10

12.10.2 Study 1 longitudinal cohort

Both urine and biopsies were collected in the longitudinal cohort.

12.10.2.1 Urine

Urine was collected at closure of the ileostomy and at 6 monthly time-points. There were 33 samples. The median age of the cohort was 39 years (16-53). There were 24 males and 9 females. Four of these patients developed PIP within a year.

12.10.2.2 Patient demographics

There were 14 patients included in the UC cohort and six patients included in the FAP cohorts. The time points collected are highlighted below with "*".

Table 37. urine time points collected.

	Patients ID	Time point 0	Time point 1	Time point 2
UC	1		*	*
	2	*		
	3	*	*	*
	4		*	*
	5	*		*
	6	*	*	*
	7	*	*	
	8	*	*	
	9	*	*	
	10	*		
	11	*	*	
	12	*	*	
	13	*		
	14		*	
FAP	1	*	*	
	2	*	*	
	4	*	*	
	5	*		
	6	*		

12.10.2.3 Biopsies

There were 56 biopsy samples. The median age of the cohort was 40 (range 20-60). There were 17 males and 5 females in the cohort. There were three patients with normal terminal ileums included. There were 15 UC patients and 6 FAP patients. The time points collected are highlighted below with "*".

Table 38. Time points collected biopsies.

	ID	Time point 0	Time point 1	Time point 2	Time point 3
UC	1	*	*	*	*
	2	*	*		
	3			*	*
	4	*			
	5	*		*	*
	6	*	*	*	*
	7	*	*	*	*
	8	*	*	*	*
	9	*		*	*
	10	*	*	*	
	11		*	*	
	12	*	*		
	13	*	*	*	
	14	*	*	*	
	15	*			
FAP	1	*	*		
	2	*	*	*	
	3	*	*		
	4		*	*	
	5		*		
	6	*	*		

12.10.3 Treatment arm

12.10.3.1 Urine

On multi-variate analysis there were no significant differences between responders and non-responders or between those on antibiotics and those off-antibiotics. This was also the case on univariate analysis where no differences in individual SCFAs were found across the cohort. (See appendix 3). I was unable to compare responders and non-responders on and off antibiotics separately, due to small numbers.

12.10.3.2 Serum

On multivariate analysis there were no significant differences between patients who responded to treatment and those that did not. There were also no significant differences between patients on and off-antibiotics. (see appendix 3). On univariate analysis (Student T-test) there were differences in isobutyric acid, acetic acid and caproic acid between responders and non-responders with significantly lower isobutyric acid (671.5 mM in responders vs 727.1 mM in non-responders (p<0.03)) and significantly higher acetic acid (625.1 mM vs 376.5 mM) in responders than non-responders (p< 0.04) who were both off antibiotics However when corrected p-values were used both were not considered significant.. see table 39.

Table 39. serum responders vs non-responders off antibiotics (concentrations in mM).

	Acetic acid	Propanoic acid	Isobutyric acid
non-responders off average	376,5	143.6	671.5
Non-responders off median	625.1	192.2	727,1
responders off average	631.6	11.90	650.1
responders off median	625.3	11.5	660.0
p-value	0.04	0.08	0.03

Table 40. serum responders vs non-responders p values.

Measurement	2-Hydroxybutyric	Lactic acid	Isobutyric acid	Acetic acid	Propanoic acid	Butyric acid	2-Methyl butyric acid	Isovolaric acid	Valeric acid	Caproic acid
Responders vs non-responders (p-value)	0.33	0.713	0.03	0.04	0.16	0.68	0.94	0.45	0.10	0.08

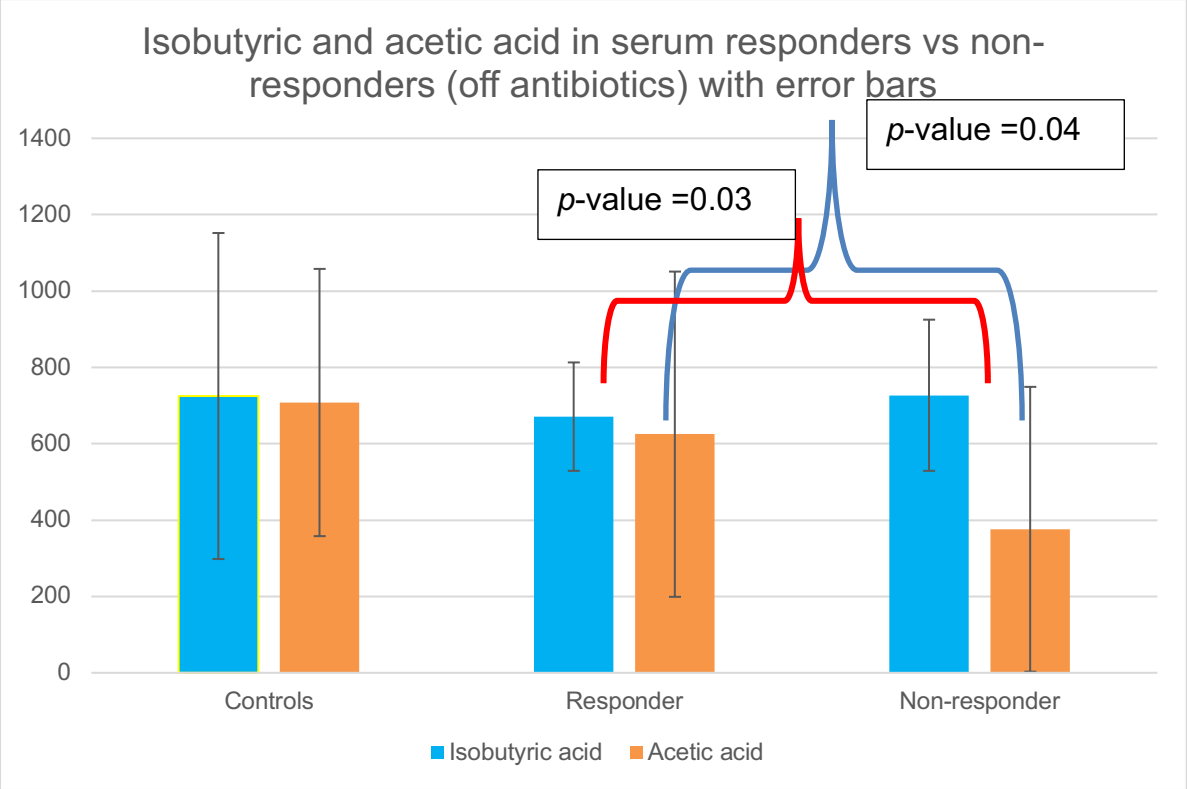


Figure 43. Isobutyric acid and acetic acid in serum responders vs non-responders (off antibiotics) with error bars.

12.10.3.3 Faeces

On multivariate analysis the OPLS-DA model did not predict the response to antibiotics. There were no significant differences on univariate models or multivariate models when comparing responders and non-responders on antibiotics or responders and non-responders off antibiotics. (See appendix 4) On univariate analysis there was no significant difference observed between Patients on and off antibiotics. No variations were observed between Responders and non-responders

12.10.4 Longitudinal cohort

12.10.4.1 Urine

A number of comparisons were made between the longitudinal samples including differences in SCFA at different time-points, as well as differences between UC patients and FAP patients. The *p*-values of the paired t-tests are summarised in table 41.

Table 41. Univariate analysis of longitudinal urine samples (red signifies a statistically significant result after Bonferonni correction).

Time point	2-hydroxybutyric acid	Lactic acid	Isobutyric acid	Acetic acid	Propanoic acid	Butyric acid	2 Methyl butyric acid	Isovaleric acid	Valeric acid	Caproic acid
UC T1 vs UC T2	0.01	0.41	0.59	<0.01	0.31	0.08	0.80	0.62	0.95	0.38
UC T1 vs UC T3	0.04	0.98	0.67	0.18	0.29	0.17	0.59	0.91	0.95	0.70
UC T1 vs FAP T1	<0.01	0.05	0.83	0.44	0.16	0.01	0.29	0.74	0.60	0.83
UC T2 vs FAP T2	0.34	0.03	0.42	0.11	0.98	0.30	0.42	0.63	0.88	0.47
T1 PIP vs non PIP	0.80	0.36	0.48	0.06	0.74	0.70	0.98	0.24	0.45	0.85

T1=At time of closure of ileostomy

T2= 6 months after closure of ileostomy

T3=12 months after closure of ileostomy

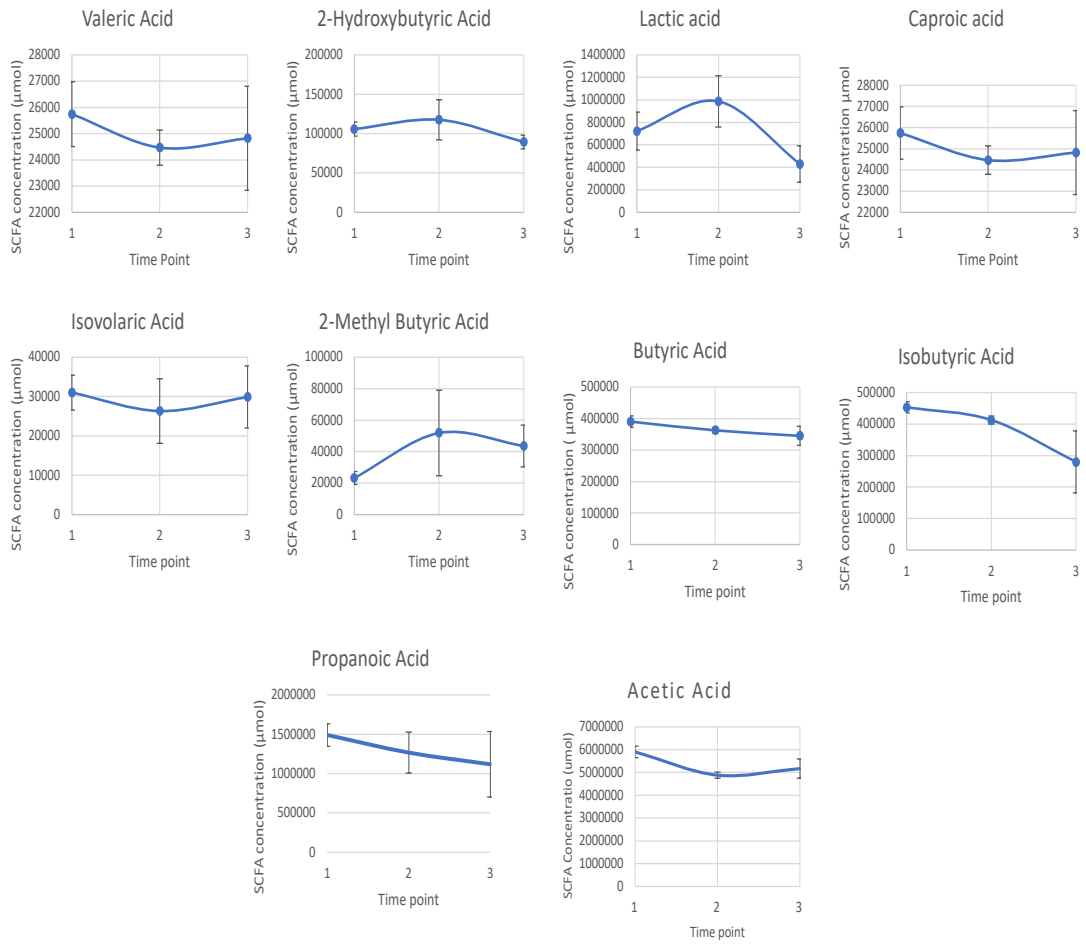


Figure 44. urine longitudinal changes in UC pouches.

The above figures show the longitudinal changes in the UC cohort with error bars. Longitudinally the majority of SCFA decreased over-time with the lowest concentrations found at time point 3 (12 months) in all but isovaleric acid and 2-methyl butyric acid. There were significant differences longitudinally in UC patients in hydroxybutyric acid and acetic acid between time point 1 and 2 (p=0.01 and <0.01 respectively) and between hydroxybutyric acid between time point 1 and 3 in the UC patients (p=0.04).

12.10.4.2 Biopsies

A number of comparisons were made between the longitudinal samples including differences in SCFA at different time-points, as well as differences between UC patients and FAP patients. The findings are summarised in Table 42.

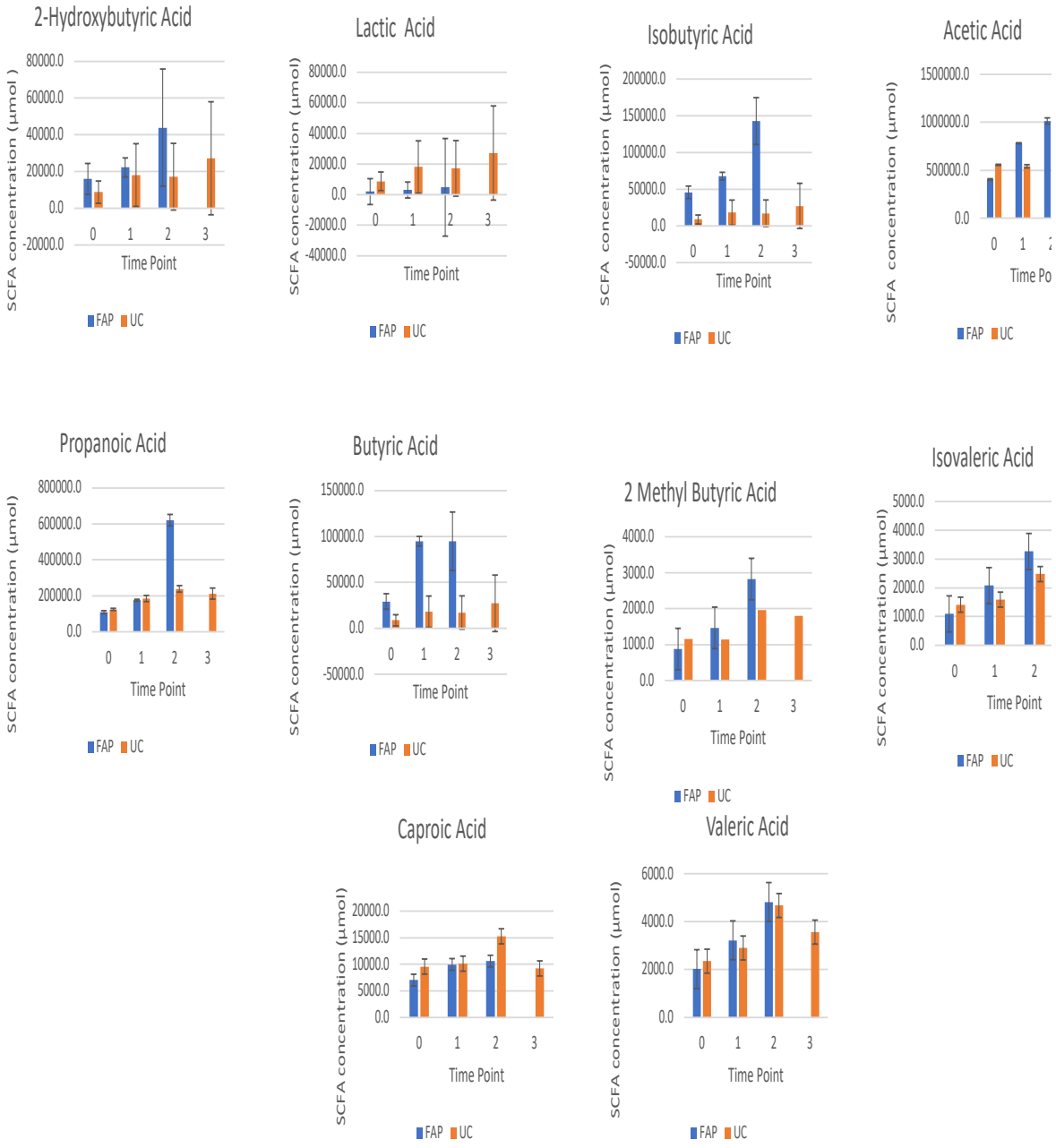


Figure 45. SCFA changes longitudinally in UC and FAP patients.

Table 42. p-values comparing SCFA levels between different time points longitudinal biopsy samples (blue signifies approaching statistical significance, red signifies a statistically significant result following Bonferroni correction).

Measurement	Cohort	2-hydroxybutyric Acid	Lactic Acid	Isobutyric Acid	Acetic Acid	Propanoic Acid	Butyric Acid	2-methyl butyric Acid	Isovaleric Acid	Valeric Acid	caproic Acid
T0 vs T1	FAP	0.77	0.34	0.75	0.18	0.36	0.34	0.33	0.27	0.25	0.50
T0 vs T2		0.58	0.58	0.59	0.57	0.53	0.58	0.55	0.50	0.58	0.73
T0 vs T2-3		0.45	0.26	0.25	0.24	0.25	0.25	0.21	0.18	0.24	0.34
T1 vs T2		0.62	0.70	0.62	0.79	0.57	1.00	0.64	0.67	0.72	0.93
T1 vs T2-3		0.42	0.44	0.28	0.40	0.30	0.84	0.30	0.37	0.44	0.60
T0 vs T1	UC	0.21	0.52	0.28	0.64	0.92	0.45	0.80	0.80	0.51	0.30
T0 vs T2		0.16	0.60	0.93	0.83	0.56	0.47	0.16	0.10	0.60	0.81
T0 vs T3		0.27	0.68	0.58	0.43	0.49	0.62	0.43	0.33	0.78	0.66
T1 vs T2		0.86	0.07	0.05	0.57	0.60	0.52	0.12	0.16	0.07	0.03
T1 vs T3		0.61	0.38	0.10	0.98	0.52	0.51	0.32	0.55	0.32	0.57
T2 vs T3		0.68	0.91	0.42	0.31	0.78	0.87	0.45	0.29	0.86	0.40
T0 PIP vs non PIP	ALL	0.77	0.80	0.56	0.82	0.57	0.61	0.66	0.78	0.80	0.81
T0 PIP vs non PIP	UC	0.80	0.93	0.60	0.98	0.63	0.69	0.75	0.91	0.93	0.92
T1 PIP vs non PIP	ALL	0.13	0.04	0.23	0.17	0.22	0.78	0.57	0.72	0.28	0.19
T1 PIP vs non PIP UC	UC	0.09	0.02	0.03	0.19	0.08	0.28	0.06	0.05	0.02	<0.01
T0	UC vs FAP	0.91	0.18	0.31	0.10	0.25	0.25	0.18	0.16	0.17	0.17
T1	UC vs FAP	0.29	0.97	0.78	0.55	0.57	0.64	0.88	0.84	0.84	0.99

T0= 2 months prior to ileostomy closure and restoration of continuity

T1=At time of closure of ileostomy

T2= 6 months after closure of ileostomy

T3=12 months after closure of ileostomy

PIP= developed PIP between 6 months to 12 months following closure of ileostomy

The most consistent changes across SCFA were found at time point 1 at the time of pouch closure. These are represented graphically below:

Develop PIP

Do not develop PIP

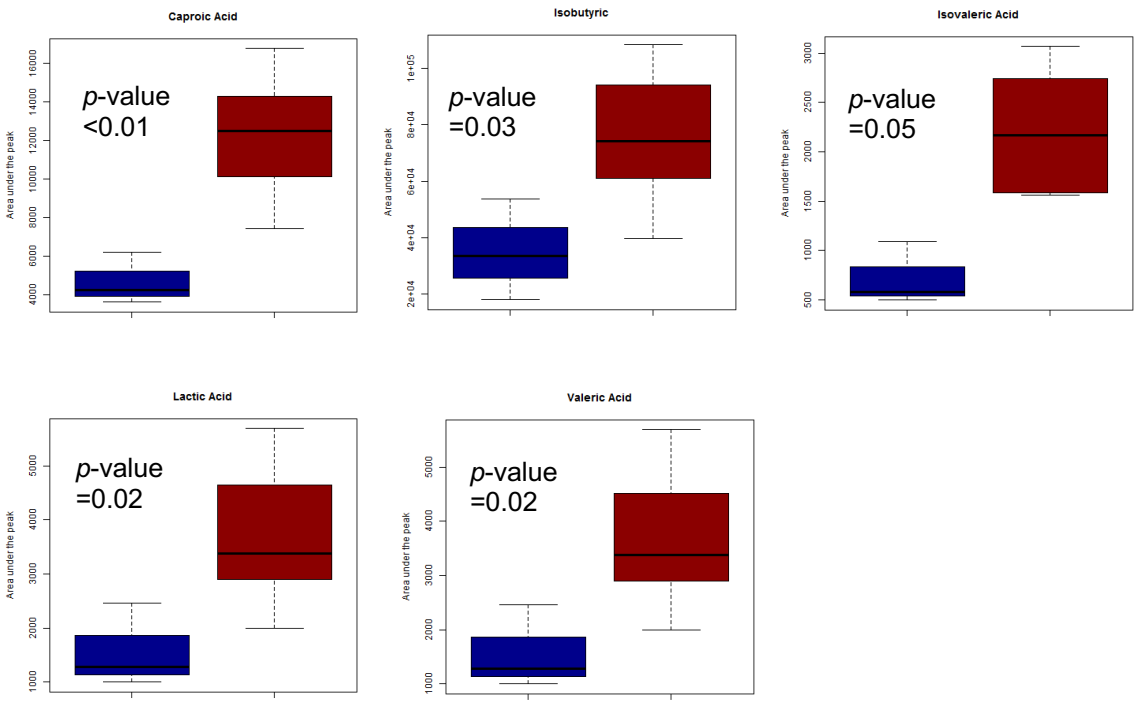


Figure 46. Box whisker plot of SCFA levels in tissue at time point 1 between those that develop PIP within 1-year vs those that do not develop PIP.

12.11 Discussion

These results suggest that the mechanism behind the success of antibiotic treatment for PIP is not strongly correlated to SCFAs in the serum, stool or urine. Furthermore, there were no significant patterns that could predict treatment response or differentiate between responders and non-responders to treatment. Interestingly however, when comparisons were made between responders and non-responders who were off antibiotics, serum acetic acid was found in significantly higher concentrations in the responders.

The longitudinal study has suggested that a decrease in SCFA at time of closure of ileostomy may predict the onset of PIP within a year. In particular caproic acid, valeric acid, isovolaric acid, isobutyric acid and lactic acid were found in lower concentrations in the tissue at time of closure of the ileostomy in those that developed PIP compared with those that did not develop PIP within a year.

Supporting the hypothesis that SCFA may have a role in the aetiology of PIP, my study highlighted that there were differences in serum samples in the SCFA isobutyric acid, acetic acid and caproic acid between responders and non-responders with lower levels of isobutyric acid in responders vs in non-responders (p<0.03) and higher levels of acetic acid in responders compared with non-responders p< 0.04. The importance of peripheral blood SCFA is well documented ,with studies suggesting that they exert an anti-inflammatory effect through G-protein coupled receptors and downstream regulation of pro-inflammatory and anti-inflammatory cytokines[435–439]. My findings therefore also support that SCFA peripherally can help distinguish between responders' and non-responders and may possibly help exert local effects on the bowel through the G-protein coupled receptors and activation of the immune system.

Previous work using faecal samples has suggested that SCFA were decreased in those that have PIP with a mean SCFA concentration of 56.2 mmol/l compared with 139.0 mmol/l in healthy controls (p<0.01). My study did not support this finding in faecal samples in either those who responded to treatment or those that did not respond to treatment. Importantly, less than 10% of total body SCFAs appear in the faeces. This therefore highlights that faecal SCFA only account for a small amount of the total body SCFA[404]. It must also be highlighted that my sample size is relatively small which may also contribute to this finding. Furthermore, we analysed SCFA in the stool using a more comprehensive technique than was used when this study was performed.

It has been shown that SCFA are overall hydrophobic molecules with low molecular weights, and in their protonated forms, acetate, propionate and butyrate can be readily absorbed via non-ionic diffusion across the apical membrane of colonocytes[411,440]. Other mechanisms of uptake into the cell include sodium-coupled monocarboxylate transporters (SCMTs) that utilize the colonic Na^+ concentration gradient[404,441]. Within this class of transporter the SLC5A8, proton-coupled monocarboxylate

transportation and SCFA-bicarbonate antiporters have also been proposed as viable mechanisms for SCFA uptake as well as regulators of lumen pH[413,442,443]. A possible explanation for my findings may be that there are defective transport mechanisms into tissues in patients who go on to develop PIP.

Limitations of this study include potential confounding factors. We did not fully account for dietary habits which are known to alter SCFA, furthermore my samples size is relatively small. Also, it is very difficult to account for other medications and lifestyle factors which are known to contribute to SCFA metabolic alterations. Ideally correlations between the tissue microbiota and SCFA levels may have given a better understanding of the interplay between the SCFA and the gut microbiota in health and disease.

Future studies should account for dietary changes including dietary intake of fibre, medications and modifiable lifestyle factors that are known to alter SCFAs. Correlations should also be made between the microbiota known to be responsible for SCFA production not just in faeces but in biopsy tissues using next-generation sequencing. Understanding the potential mechanisms that SCFA work on a tissue and cellular level may also enhance my understanding of the role of SCFA in PIP.

12.12 **Conclusion**

SCFA in human pouch tissue levels at time of closure of the ileostomy may predict the development of PIP within 1 year. It is plausible that SCFA levels in the tissues are important in the aetiology of PIP. Interestingly SCFA levels in the serum also showed significant differences between responders and non-responders suggesting SCFA have a role in the development of PIP beyond the local environment.

Summary and future work

The current treatment of chronic PIP for many patients remains sub-optimal and in many cases are associated with considerable morbidity which impacts significantly on a patient's quality of life. It is important to note that the ileoanal pouch can be considered a 'quality of life operation' and hence for many patients who suffer with chronic PIP, the operation may cause a decrease in quality of life rather than improve it.

As part of this research it was important to assess quality of life in patients with an ileoanal pouch. As an ongoing development from this research we have set up a multi-centre national study with the aim of addressing this called the "Study of Quality of Life and Healthcare Utilisation in Patients with Ulcerative Colitis Undergoing Ileal Pouch Surgery: Prospective Observational Study" (SOQCS). This uses an online portal and recruits' patients who have undergone RPC or have previous undergone RPC and captures a variety of quality of life measures longitudinally every six months for up to three years. Currently we are at the stage where we are recruiting patients and this project will help address the quality of life question s regarding RPC.

This thesis has supported the notion that the gut microbiota may be implicated in maintenance of health and disease of the ileoanal pouch. This thesis has suggested that it may be possible to predict those patients that will develop PIP using measurements of SCFA. SCFA levels in tissues may be potentially modifiable. SCFA are produced by SCFA producing bacteria and therefore if these can be increased it may be possible to prevent PIP. It is also important to note that SCFA are derived from insoluble fibres that are dietary components and therefore dietary interventions may be a therapeutic option to prevent and treat PIP. Lastly specific SCFA enemas have previously been utilised in the clinical setting and could be modified and optimised to potentially help PIP patients. Overall it was found that SCFA increased longitudinally in both FAP and UC patients suggesting that the pouch perhaps adapts over time to increase these levels. Furthermore, FAP patients tended to have higher levels of individual SCFA when compared with UC patients at each time point. As the incidence of PIP is different between these two groups it gives further evidence that the SCFA may be important in pathogenesis of PIP.

Future studies should therefore validate the findings and try and establish a link between the SCFA levels in the tissue, faeces and correlate that with diet and the SCFA producing microbiota. On a clinical level pilot studies addressing methodologies of increasing SCFA through diet, microbiota manipulation and SCFA therapies may elucidate their role. Future studies should use an integrative multiomics level approach to establish how the environment, immune system and the metabonomics interconnect to maintain health and disease in a pouch.

Furthermore, tools that are used to measure PIP have yet to be validated and may not therefore be sensitive and specific enough to separate responders' vs non-responders. It is therefore imperative that for future studies that validate these tools.

Weaknesses of this thesis is the relatively small sample numbers which makes data interpretation difficult. Furthermore, we could not correlate longitudinal pouch biopsy SCFA with stool SCFA which may have allowed us an insight into the mechanistic ways they contribute to health and disease.

Conclusion of thesis

Inflammation within the pouch can often be difficult to treat and associated with much morbidity for many patients. This thesis has enabled me to explore the current treatments for inflammatory pouch pathologies whilst also exploring some of the novel therapeutic avenues. For some patients the established treatments culminate in relief of symptoms, however for others the current medical therapies remain sub-optimal. This body of work has highlighted that there are some potential underlying mechanistic pathways that may lead onto inflammation and by understanding these pathways it seems possible that for some patients we can improve symptoms . This work has also suggested to me that pouchitis and chronic pouchitis are likely to be a group of heterogenous diseases that may require slightly different treatment approaches based on the underlying pathway that is driving the inflammation. Through understanding how the genetics, diet and environment interact and using integrated ‘omic’ technologies I believe we may be able to achieve a more personalised treatment algorithm for patients with inflammatory pouch problems and ultimately enable a better quality of life for these patients.

Appendix 1

Institution constipation/evacuation disorder

Biofeedback Assessment Sheet

Therapist:
Date:

Hospital Label:

Telephone: Home
Work

Referrer:

GP:
Marital status: single/married/lives with partner/divorced/widowed

Occupation:

Ethnic Group: White/Asian/black/Chinese/other

Transit: Slow/Normal/not done
SRUS/megarectum/rectocele/prolapse/constipation/evacdis

Start of Problem:
Main problems now:
Urge: yes/no/sometimes abdo/rectum
Frequency 4-8 times daily Longest bno:
Consistency :BSFS NO;
Blood: Yes/No/Sometimes
Bright/Dark/on wiping/in stool/in toilet
Haemorroids/anal fissure
Mucus: yes/no/sometimes

Digitate: yes/no sometimes

Incomplete: yes/no/sometimes

Strain: Yes/no/sometimes

Pain: Yes/no/sometimes

Bloating: Yes/no/sometimes

Laxatives: Yes/no/sometimes

Other meds:

PMH/Surgery:

Continence: Passive/urgency/post defaecation/bladder

Pregnancies: yes/no para difficult deliveries

Bowel pathology excluded:

History of depression

Diet: regular? Example (if relevant)

Fluids per day

Effect on lifestyle/relationships/emotions

Appendix 2

Urine Longitudinal comparisons

Comparison	Model type	Scaling	Components	Number of samples included	R^2x (cum)
All samples with QC	PCA-X	UV	2	69	0.192
All samples with QC	PCA-X	PAR	2	69	0.385
Al samples without QC	PCA-X	UV	2	66	0.196
All samples without QC	PCA-z	Par	2	66	0.196

Appendix 3

NMR Urine all samples

Comparison	Model type	Scaling	Components	Number of samples included	R^2X (cum)
All samples with QC	PCA	UV	2	69	0.192
All samples with QC	PCA	PAR	2	69	0.385
Al samples without QC	PCA	UV	2	66	0.196
All samples without QC	PCA	Par	2	66	0.196

A3.1 Urine Longitudinal

Comparison	Model type	Scaling	Components	Number of samples included	R^2X (cum)	R^2Y(cum)	Q^2(cum)	*P*-value	Notes
All longitudinal samples	PCA	UV	2	36	0.25		0.112		
Male vs female	PLS	UV	2	36	0.229	0.873	0.582	3.33E-05	Model explored
male vs female	PLS	PAR	2	36	0.261	0.814	0.546	5.19E-05	
ALL developed PIP vs no PIP	PLS-DA	UV	2	36	0.196	0.858	0.198	0.440	
ALL developed PIP vs no PIP	PLS-DA	PAR	2	36	0.22	0.768	0.169	0.477	
FAP T1 vs UC T1	PLS-DA	UV	2	21	0.243	0.867	0.387	0.500	
FAP T1 vs UC T1	PLS-DA	PAR	2	21	0.688	0.864	0.491	0.070	
FAP T2 vs UC tT2	PLS-DA	UV	3	14	0.323	0.977	0.624	0.217	
FAP T2 vs UC tT2	PLS-DA	PAR	4	14	0.767	0.944	0.738	0.550	
UC t1 vs UC t2	OPLS	UV	2+9	29	0.6	1	0.291	0.989	
UC t1 vs UC t2	OPLS	PAr	2+2	29	0.618	0.677	-0.0551	1	
FAP T1 vs t2	OPLS	UV	1+1	10	0.243	0.995	-0.046	1	
Age of pouch	PLS	UV	2	36	0.215	0.826	0.388	0.00354966	Despite significance model not strong enough
Age of pouch	PLS	PAR	2	36	0.239	0.724	0.347	0.00948808	Despite significance model not strong enough

Comparison	Model type	Scaling	Components	Number of samples included	R^2X(cum)	R^2Y(cum)	Q^2(cum)	*P*-value	Notes
All developed PIP vs no PIP t1	OPLS	uv	3+0+0	18	0.278	0.783	0.00985	0.952036	
All developed PIP vs no PIP t1	OPLS	PAR	1+0+0	18	0.141	0.325	-0.0255	1	

A3.2 Urine treatment

Comparison	Model type	Scaling	Components	Number of samples included	R^2X(cum)	R^2Y(cum)	Q^2(cum)	*P*-value
All samples	PCA	UV	2	26	0.234		0.00747	
Male vs female	PLS-DA	UV	2	26	0.149	0.933	-0.0176	1
Male vs female	PLS-DA	PAR	2	26	0.249	0.705	0.00841	1
Responder vs non responders including controls	PLS-DA	UV	3	26	0.23	0.878	0.247	0.600414
responder vs non responder excluding controls	PLS-DA	UV	2	21	0.19	0.952	0.532	0.05
responder vs non responder excluding controls	PLS-DA	PAR	2	21	0.25	0.859	0.304	0.05
non responder vs controls	PLS-DA	UV	2	17	0.139	0.982	-0.026	0.532
On vs off antibiotics	OPLS-DA	UV	1+1	22	0.102	0.453	0.057	1
On vs off antibiotics	OPLS-DA	PAR	1+1	22	0.259	0.744	-0.454	1
On antibiotics responder vs non responder	OPLS-DA	UV	1+1	9	0.039	0.997	0.67	0.26
On antibiotics responder vs non-responder	OPLS-DA	PAR	1+1	9	0.562	0.932	0.712	0.20

A3.3 Urine Responder *vs* non-Responder excluding controls

Comparison	Responder *vs* non-Responder excluding controls
Model type	PLS-DA
Scaling	UV
Components	2
samples	21
R^2x (cum)	0.19
R^2Y(cum)	0.952
Q^2(cum)	0.532
p value	0.05

A3.4 Serum responders vs non-responders

Comparison	Model type	Scaling	Components	Number of samples included	R^2X (cum)	R^2Y(cum)	Q^2(cum)	*P*-value
ALL samples	PCA	UV	2	30	0.22		0.0824	
All samples without QC	PCA	UV	2	27	0.23		0.0729	0.79
responders vs non responders	OPLS-DA	UV	1+1+0	22	0.199	0.963	0.0904	0.87
responders vs non responders	OPLS-DA	PAR		22	0.289	0.545	-0.265	1
non responders vs controls	OPLS-DA	UV	1+1+0	18	0.218	0.979	0.084	1
non responders vs controls	OPLS-DA	PAR	1+1+0	18	0.637	0.61	-0.553	1
on vs off abx	OPLS-DA	UV	1+1+0	22	0.193	0.955	0.0852	0.808802
On vs off abx	OPLS-DA	PAR	1+1+0	22	0.659	0.362	-0.296	1

Appendix 4

Multivariate analysis of faecal samples responders vs non responders

comparison	A	Samples numbers	Model type	Scaling	R2(x)	R2(y)	Q2	Cv-anova
responders vs non responders	2	28	PCA-x	UV	0.713		-0.21	
responders vs non responders	2	28	PCA-x	PAR	0.861		0.0759	
responders vs non responders	1+0+0	28	OPLS-DA	UV	0.354	0.0539	-0.0197	1
responders vs non responders	1+0+0	28	OPLS-DA	PAr	0.827	0.0603	-0.0374	1
Non responders vs controls	2	26	PCA-x	uv	0.73		-0.21	
Non responders vs controls	2	26	PCA-x	PAr	0.836		0.0908	
Non responders vs controls	1+1+0	26	OPLS-DA	UV	0.588	0.177	-0.302	1
Non responders vs controls	1+1+0	26	OPLS-DA	PAR	0.75	0.169	-0.11	1
Responder vs controls	2	12	PCA-x	uv	0.628		0.00716	
Responder vs controls	2	12	PCA-x	PAr	0.739		0.0917	
Responder vs controls	1+1+0	12	OPLS-DA	UV	0.536	0.373	-0.685	1
Responder vs controls	1+1+0	12	OPLS-DA	PAR	0.731	0.311	-0.26	1
On vs off antibiotics	2	28	PCA-x	uv	0.713		-0.21	
On vs off antibiotics	2	28	PCA-x	PAr	0.861		0.0759	
On vs off antibiotics	1+1+0	28	OPLS-DA	UV	0.628	0.222	-0.161	1
On vs off antibiotics	1+1+0	28	OPLS-DA	PAR	0.786	0.177	-0.267	1

Appendix 5

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1. J P Segal, N S Ding, G Worley, S Mclaughlin, S Preston, O D Faiz, S K Clark, A L Hart: *Systematic review with meta-analysis: The management of chronic refractory PIP with an evidence-based treatment algorithm*. Alimentary Pharmacology & Therapeutics 12/2016; 45(5):n/a-n/a., DOI:10.1111/apt.13905

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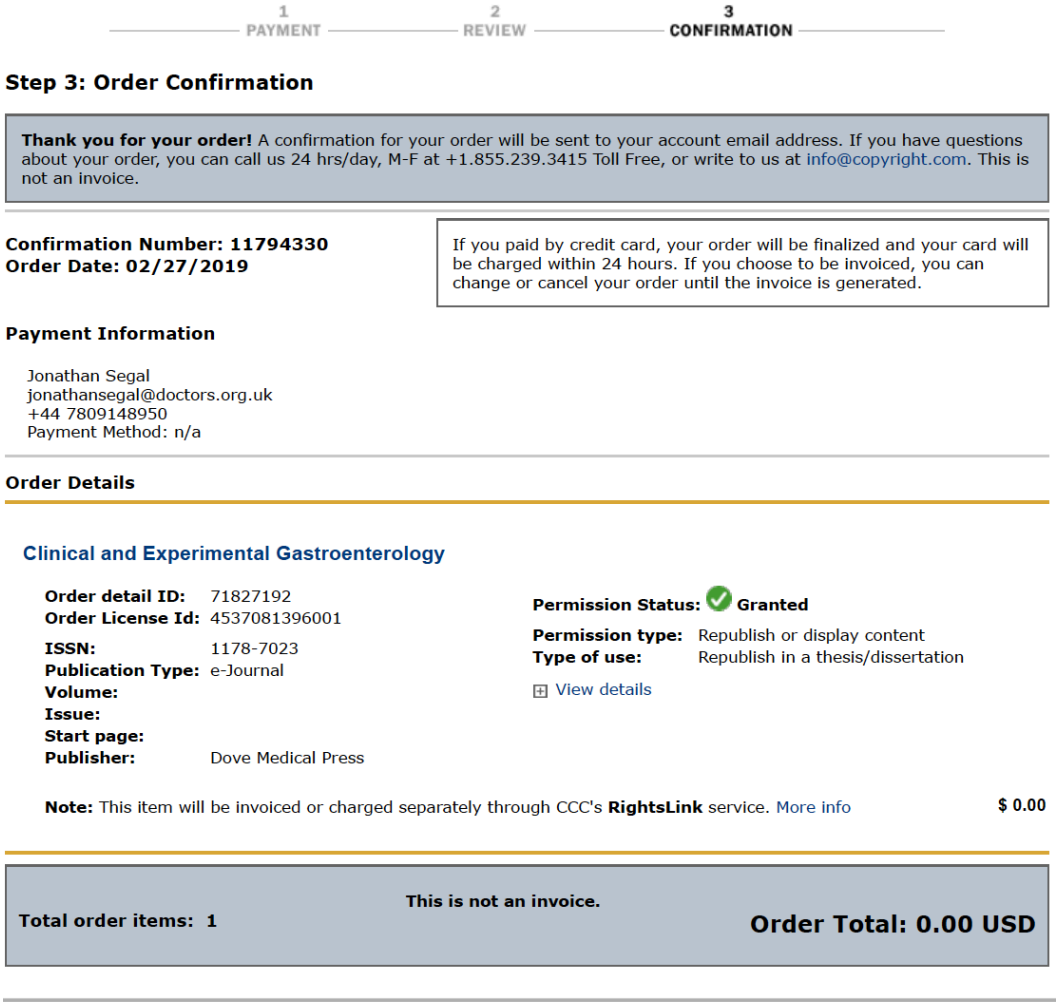

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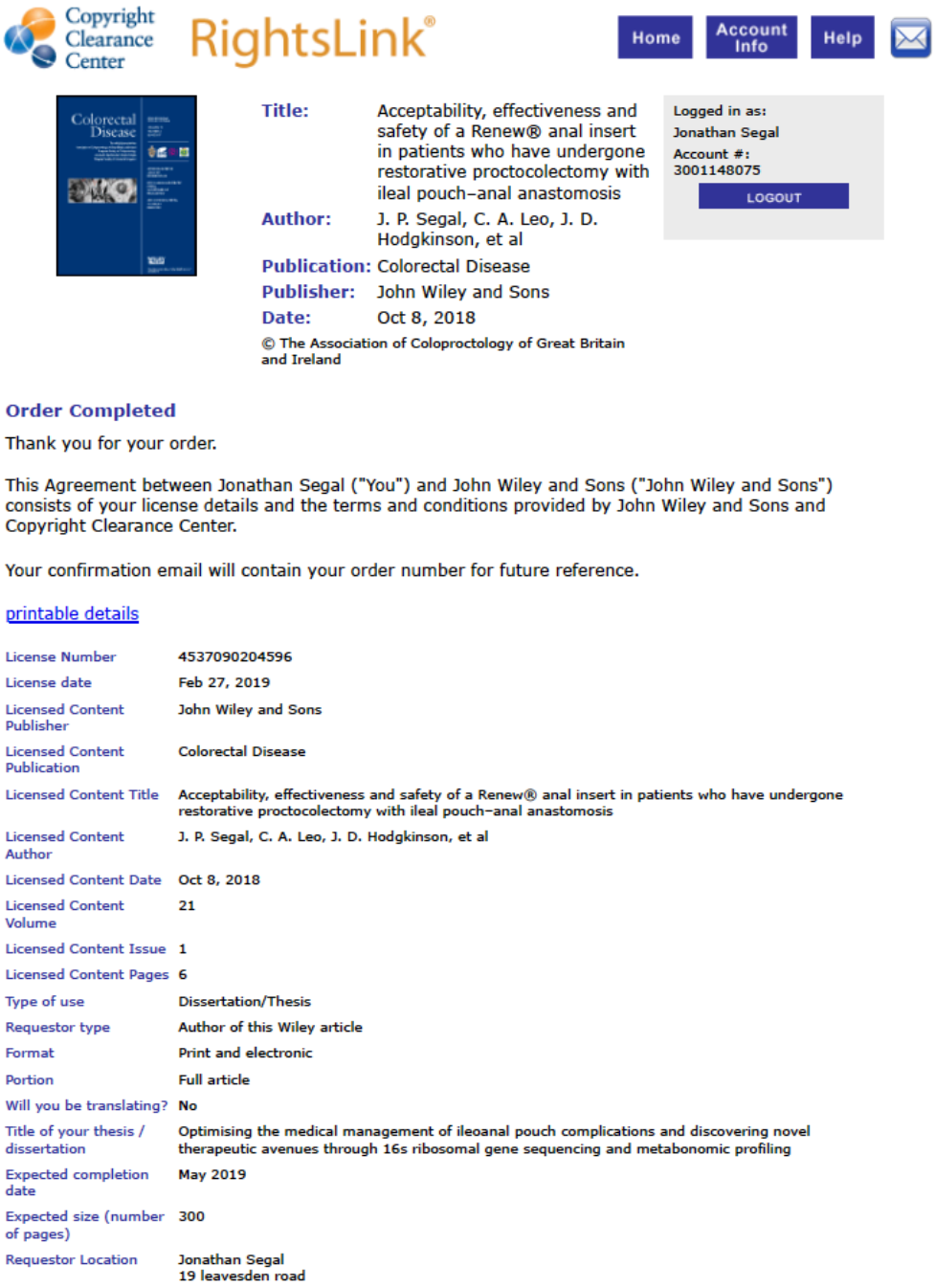

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