Immunological and Microbiological Studies in Post-operative Crohn's Disease

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Abstract

Crohn's disease is a chronic, inflammatory condition of the bowel. The aetiology of Crohn's has not been fully elucidated, but is believed to arise from the interaction between the gut microbiota, the host immune system and environmental factors. A majority of Crohn's disease patients will require a bowel resection as a result of disease, which causes significant morbidity and impacts on quality of life. While surgery can ameliorate clinical symptoms, the disease often initially recurs at the site of the resection. This may be sub-clinical initially, and can be identified endoscopically.

This thesis investigates the immunological and microbiological characteristics of post-operative Crohn's disease recurrence in, addressing serologic markers, the faecal microbiome and the possible contribution of *Proteus* species to gastrointestinal disease.

Serologic markers, while disappointing for prediction of disease recurrence, did have some utility for identifying patients at the highest risk of disease recurrence. I also demonstrated lower rates of antibody positivity in Crohn's patients who smoke for the first time, indicating that these results should be interpreted with caution in current and past smokers.

I have further evaluated the faecal microbiome in the setting of post-operative disease using metagenomic techniques. This work showed that after resection for Crohn's disease, enrichment of the bacterial family *Lachnospiraceae* is associated with maintenance of disease remission, while patients enriched for the *Enterobacteriaceae* are more likely to recur. Recurrence may result from a higher abundance of enteric pathobionts such as *Proteus*, *Klebsiella, Serratia*, and *Escherichia*. The *Lachnospiraceae* are an important family of butyrate producing bacteria in the gut, and depletion of this bacterial family may perpetuate the expansion of *Enterobacteriaceae* via environmental perturbation and ecologic shifts. These findings indicate possible protective and pathogenic bacteria in post-operative recurrence.

Finally, I addressed the potential contribution of *Proteus* species (from the *Enterobacteriaceae* family) to gastrointestinal diseases including to inflammatory bowel disease. *Proteus* spp. are low-abundance commensals of the human gut that harbour significant pathogenic potential. Preliminary evidence of a pathogenic role in the gut should stimulate further investigation.

I have elucidated some aspects of the microbiome and host immune factors involved, in the pathophysiology of post-operative Crohn's disease recurrence. This work should encourage further work on a unifying hypothesis for the aetiology of disease recurrence after resectional surgery for Crohn's disease.

Declaration

This is to certify that this thesis:

i) contains no material that has been accepted for the award of any other degree or diploma in any university of other institution,

ii) comprises only my original work except where indicated in the Preface,

iii) due acknowledgement has been made in the text to all other material used,

iv) is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices

Amy L. Hamilton, October 2017

Preface

The work presented in this thesis was undertaken under the principal supervision of Professor Michael A Kamm, Department of Gastroenterology, St Vincent's Hospital and Department of Medicine, University of Melbourne.

The laboratory work was initially co-supervised by Associate Professor Carl Kirkwood, Director, Enteric Virus Group, Murdoch Children's Research Institute, Parkville, Australia. From September 2015 this work was co-supervised by Professor Mark Morrison, Diamantina Institute, University of Queensland, Australia and A/Prof Michael Inouye, Systems Genomics Group, Baker Heart and Diabetes Institute, Melbourne, Australia; Department of Pathology, University of Melbourne, Australia; Department of Public Health and Primary Care, University of Cambridge, Strangeways Research Laboratories, Cambridge, United Kingdom; School of BioSciences, University of Melbourne, Victoria, Australia.

Data presented herein are derived from serum and faecal samples as well as the clinical data collected as part of the Post-operative Crohn's Endoscopic Recurrence (POCER) study, designed and conducted by Dr Peter De Cruz and Professor Michael Kamm. Amy Hamilton was the Clinical Scientist assigned to this project, and was also involved in the data collection and project management prior to commencement of this PhD. Kathryn Ritchie and Efrosina Krejany assisted with data collection, monitoring and study coordination.

Others have contributed to the work presented in this thesis as detailed below:
Professor Danny Liew and Alexandra Gorelik provided some assistance with the statistical analysis undertaken within chapter 3 – Serologic antibodies in relation to outcome in postoperative Crohn's disease. Dr. Fabiyola Selvaraj performed the serologic testing as an employee of Prometheus Laboratories, San Diego, USA and the collaboration was overseen by Dr. Fred Princen. All other co-authors enrolled patients into the POCER Study, and reviewed the manuscript only.

Dr Josef Wagner and Dr. Hai Feng undertook the DNA extractions and sequencing of the faecal samples analysed in chapter 4 –The Faecal Microbiome in Post-Operative Crohn's Disease. All other work, including the quality control of sequencing data was undertaken by Amy Hamilton under the supervision of Dr Shu Mei Teo, Systems

Genomics Group, Baker Heart and Diabetes Institute, Melbourne, Australia; Department of Public Health and Primary Care, University of Cambridge, Strangeways Research Laboratories, Cambridge, United Kingdom; Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Australia. Dr Teo also taught me how to use the R platform and assisted and supervised the cleaning of sequencing data and bioinformatic analysis work presented in Chapter 4.

Professor Mark Morrison (as above) and Professor Siew Ng, Institute of Digestive Disease and Department of Medicine and Therapeutics, State Key Laboratory of Digestive Disease, Li Ka Shing Institute of Health Sciences, CUHK Shenzhen Research Institute, The Chinese University of Hong Kong, Hong Kong, China assisted with academic review of chapter 5 - *Proteus* as Putative Gastrointestinal Pathogens - A Systematic Review.

These studies were partly funded by the National Health and Medical Research Council (Dora Lush Post-graduate Scholarship GNT1093089 [myself] and a past Practitioner Fellowship [MA Kamm]) and the St Vincent's Foundation. Support for the clinical research was also received from AbbVie, The Helmsley Charitable Trust, The Gutsy Group, Gandel Philanthropy, Angior Foundation, Crohn's Colitis Australia, and the Murdoch Children's Research Institute.

Acknowledgements

I would like to thank my primary supervisor Professor Michael Kamm for his support, guidance and supervision over the course of this project. We have had a long, successful and most importantly, enjoyable working relationship over many years. I am indebted to you for your enthusiasm, mentorship and advice.

I am grateful for the contribution and support of my co-supervisors, Professor Mark Morrison and Associate Professor Mike Inouye who assisted greatly with portions of this work. Professor Carl Kirkwood and Dr. Feng Hai from Murdoch Children's Research Institute also provided invaluable support. I would also like to acknowledge the guidance of Dr. Shu Mei Teo who was instrumental to the metagenomics portion of this work. Shu Mei and Mike, I made many friends working as part of your research group and I thank you all for your support.

Dr. Peter De Cruz, thank you for your contribution to the POCER Study, without your dedication I would not have had data to analyse. This project stands on the shoulders of those who came before me, and I am indebted to Peter, Kathryn Ritchie, Soula Krejany and Dr. Emily Wright for their support and dedication to the POCER Study over the years. I'd also like to thank the patients who participated in the POCER study and gave their time (and small pieces of themselves) in the pursuit of science and better treatments for Crohn's disease. I truly hope this work does justice to your contributions.

Funding for this project was gratefully received from the National Health and Medical Research Council, through the Dora Lush post-graduate scholarship. I would also like to again thank Professor Kamm for his amazing assistance with funding through the Australian Gastro Intestinal Research Foundation, which made it possible for me to undertake this project. Further funding for portions of this project was appreciatively received from AbbVie, The Helmsley Trust, Gutsy Group, Gandel Philanthropy, Angior Foundation, Crohn's Colitis Australia and the Murdoch Children's Research Institute.

My wonderful colleague Dr. Amy Wilson-O'Brien has made it possible for me to take the time away from other duties to complete this project. Amy, your invaluable friendship, sense of humour and wonderful support will not be forgotten.

To the staff, nurses and consultants of the St Vincent's Hospital Gastroenterology Department, there are far too many people who have contributed to this to thank individually. I am very fortunate to work in such a happy, supportive and research-

focused department. Special thanks must go to Professor Alex Thompson (chair of my academic committee and Head of Department) for his support, and to Dr. Sally Bell for providing a sympathetic ear. As a scientist embedded in a hospital department, I am heartened that I am able to make a small contribution to a clinical department that provides such amazing patient care. I thank you all for your encouragement and support.

I would also like to acknowledge editing assistance provided by the talented Jonathan Ridler, who was kind enough to read my thesis and provide such valuable feedback.

I am blessed to have wonderful family and friends who have all come on this journey with me, including all the IBD nurses, Chamara, Julien, the Briggs family, Sam, Marcus, Jake, Artika, JJ, Zina, Lori and many others. I can't thank you all individually, but I am in your debt. Kathy, I hope you will be next to graduate. Noah, Elijah and Indy, you deserve a special mention for all your love and support, and for looking after Dad in my absence.

To my amazing parents, Robert and Susan…

This PhD has coincided with illness and difficult times, and even through everything you have been unwavering in your love and support of me. I know I may not have made it easy, but your belief in me will never be forgotten. Your contribution to this is far more than you will ever know, not least instilling in me a sense of curiosity and lifetime love of learning. *Dad, this one is for you!*

Finally, a PhD is never undertaken alone…

To my long-suffering partner Ian, thank you from the depths of my heart. You have held the fort at home while I worked long hours, listened to me when things were tough, and been a wonderful, loving part of my life for many years. Thank you for your humour and compassion, and for keeping me (mostly) sane though this process. You have unwaveringly supported my crazy life choices, and I truly could not have started or finished this without you. I love you very much, and I look forward to many more happy years together.

Amy Hamilton, October 2017

List of Publications and Abstracts Relating to this Thesis

Published Articles

Hamilton AL, Kamm MA, De Cruz P, *et al*. Serologic antibodies in relation to outcome in postoperative Crohn's disease. Journal of Gastroenterology and Hepatology 2017;32:1195-1203.
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Articles Submitted for Publication

Hamilton AL, Kamm MA, Ng SC, Morrison M. *Proteus* as Putative Gastrointestinal Pathogens - A Systematic Review

Submitted, reviewed and under revision at Clinical Microbiology Reviews.

Conference Abstracts

European Crohn's and Colitis Organisation (ECCO) Congress 2015

Poster Presentations

Hamilton AL, Kamm MA, Selvaraj F, *et al*. P207. Serological Antibodies for the Prediction of Post-operative Recurrent Crohn's Disease. Results from the POCER study, In European Crohn's and Colitis Organisation Congress 2015, Barcelona, Spain, 2015-02-01 00:00:00, 2015.

Hamilton AL, Kamm MA, Selvaraj F, *et al.* P160. Need for Recurrent Resection and Complex Phenotype are Associated with a Specific Antibody Signature in Crohn's Disease Patients undergoing Resection. Smoking is Not Associated with Prevalent Antibodies. Prognostic and Mechanistic Implications, In European Crohn's and Colitis Organisation Congress 2015, Barcelona, Spain, 2015.

Digestive Diseases Week (DDW) 2015

Poster Presentation

Hamilton AL, Kamm MA, Selvaraj F, *et al*. Sa1143 Need for Recurrent Resection and Complex Phenotype Are Associated With a Specific Antibody Signature in Crohn's Disease Patients Undergoing Resection; Smoking Is Not Associated With Prevalent Antibodies; Prognostic and Mechanistic Implications, In Digestive Diseases Week 2015, Washington D.C, USA, 4/1/April 2015, Elsevier Inc., 2015.

Oral Presentation

Hamilton AL, Kamm MA, Selvaraj F, *et al*. 597 Serological Antibodies for the Prediction of Post-Operative Recurrent Crohn's Disease Results From the POCER Study, In Digestive Diseases Week 2015, Washington D.C., USA, 4/1/April 2015, Elsevier Inc., 2015.

European Crohn's and Colitis Organisation (ECCO) Congress 2017

Poster Presentation

Hamilton AL, Kamm MA, Teo SM, *et al*. P773 Post-operative Crohn's disease recurrence is associated with specific changes in the faecal microbiome - potential pathogenic and protective roles, In European Crohn's and Colitis Organisation Congress 2017, Barcelona, Spain, Feb 01, 2017.

Australian Gastroenterology Week 2017

Oral Presentation

Hamilton AL, Kamm MA, Teo S-M, et al. Post-operative Crohn's disease recurrence is associated with specific changes in the fecal microbiome: Potential pathogenic and protective roles. Journal of Gastroenterology and Hepatology 2017;32, S2:117-118.

Prizes and Awards

Senior Medical Staff Prize for Best Basic Science Poster

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List of Other Related Publications Completed During this Candidature

De Cruz P, Kamm MA, **Hamilton AL**, *et al*. Crohn's disease management after intestinal resection: a randomised trial. The Lancet 2015;385:1406-1417.

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Wright EK, Kamm MA, Wagner J, Teo SM, Cruz P, **Hamilton AL** *et al*. Microbial Factors Associated with Postoperative Crohn's Disease Recurrence. Journal of Crohn's and Colitis 2017;11:191-203.

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1 Introduction, Background and Project Design

Crohn's disease is a chronic inflammatory bowel condition with an annual incidence of 17.4 per 100,000 Australians.[1] The cause of Crohn's disease is unknown. It is hypothesised that interactions between the intestinal microbiota, immunity, environmental factors and host genetics drive the disease process.[2]

Up to 80% of patients with Crohn's disease require intestinal resection at some stage in their disease process, and; of these 75% of patients develop post-operative recurrence which requires further surgery.[3, 4] Risk factors reported previously for earlier post-operative recurrence include smoking, previous surgery, and penetrating disease.[5]

We have previously undertaken a randomised clinical trial to assess the value of structured pre-operative assessment to establish risk of disease recurrence, followed by a regime of selective prophylactic immunosuppression. The Post-Operative Crohn's Endoscopic Recurrence (POCER) study was undertaken in 17 hospitals around Australia and New Zealand and recruited 174 patients who were then monitored for 18 months post-operatively.[6] Endoscopic evaluation was performed early to identify patients with evidence of disease, who then had intensified drug therapy. Patients provided longitudinal blood, serum and stool samples for further study of the genetic, immunologic and microbial aetiology of Crohn's disease.

We aim to develop a more sophisticated approach to risk stratification using serological, microbial and clinical tools. This will improve monitoring and treatment algorithms for post-operative Crohn's Disease patients.

Analysis of the clinical outcomes of this study have already demonstrated a reduction in recurrence rates in patients who participated in the post-operative care algorithm, as well as the utility of monitoring disease using faecal biomarkers.[6, 7] This PhD program continues the scientific and laboratory analysis of this patient cohort.

1.1 Crohn's Disease and Surgery

1.1.1 Introduction

The natural history of Crohn's disease involves a relapsing and remitting course by which intestinal damage is accumulated over time.[8] Disease activity and progression, with or without treatment, leads to the development of complicated disease and perpetuation of the inflammatory process. Complicated disease is defined as progression from an inflammatory phenotype to the development of strictures and/or penetrating disease (defined as free-perforation, acute or sub-acute perforation and/or fistulisation) (Figure 1.1).[9, 10] This variation in phenotype influences the rate of disease progression and risk of surgical intervention. This accumulated damage often results in the need for a bowel resection to remove the active disease, which improves quality of life but is not curative.[11, 12]

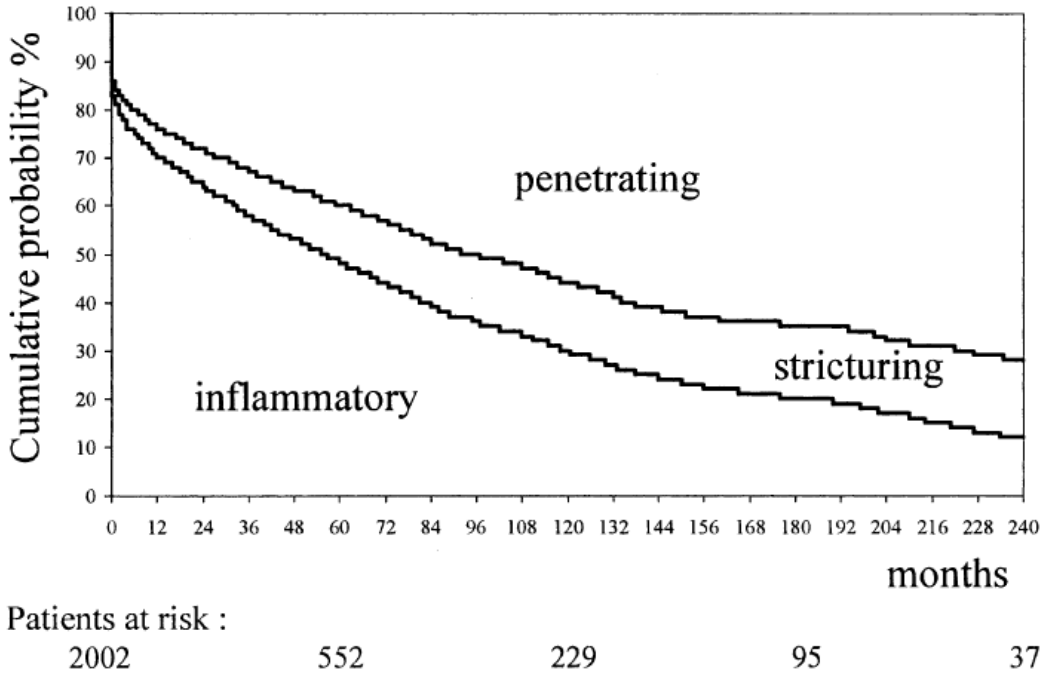


Figure 1.1 The changing phenotype of Crohn's disease over time. This Kaplan-Meier curve demonstrates the cumulative probability of developing disease complications (stricturing or penetrating phenotype) from time of diagnosis in 2002 patients. With permission from [13]

Crohn's disease phenotype is most often defined by the Montreal Classification (Table 1.1), addressing age at diagnosis, disease location and behaviour as well as defining additional disease processes such as perianal or upper gastro-intestinal disease.[10]

Age at Diagnosis	A1	≤16 years of age
	A2	17 – 40 years of age
	A3	> 40 years of Age
Disease Location	L1	Terminal ileal disease
	L2	Colonic disease
	L3	Ileo-colonic disease
	L4	Upper gastrointestinal disease
Disease Behaviour	B1	Non-stricturing, non-penetrating disease ("inflammatory disease")
	B2	Stricturing disease
	B3	Penetrating disease
Perianal Disease Modifier	+P	+ Perianal involvement

Table 1.1 Montreal Classification of Crohn's Disease.[10]

1.1.2 The Need for Surgery in Crohn's Disease

The need for resectional surgery in Crohn's disease is a large contributor to the morbidity of the condition. There are a number of possible indications for first surgery in Crohn's disease as shown in Table 1.2.[14] Burnell *et al* (2000) reported 44% of patients require surgery within one year of diagnosis when the acute and untreated disease process can lead to bowel obstruction or fistulisation.[5] Population-based studies and inception cohorts routinely demonstrate surgical rates of 10-35% in the first year; however, with improved treatments such as immunomodulators (thiopurines) and anti-TNF therapy these rates are falling. [14, 15]

Indications for First Surgery in Crohn's Disease
Failure of medical therapy
Perforation or fistulisation of the bowel including development of a phlegmon
Abdominal abscesses/sepsis
Symptomatic intestinal obstruction
Severe perianal involvement /fistulising disease
Dysplasia or neoplasia
Bleeding/anaemia
Masses or polyps visible on imaging studies
Toxic megacolon
Intestinal ischemia or necrosis

Table 1.2 Indications for initial surgery in Crohn's Disease.[16, 17]

Over time from disease diagnosis, the cumulative risk of surgery rises. A number of studies have demonstrated rates of surgical intervention between 39% - 80% in patients with a disease history of 15 years or more, with rates of resection rising with years since diagnosis.[14] A review of a U.S. Inflammatory Bowel Diseases registry demonstrated that in a population of Crohn's disease patients with a median disease duration of 12 years, nearly 70% of patients had had one or more bowel resections, with 25% requiring >2 surgical interventions.[8] Data from the IBSEN study, which followed a Norwegian population cohort for >10 years, demonstrated a surgical rate of 38%, with 9% of the included patients requiring two or more operations.[18] There is evidence that the rates of surgical intervention are falling in Crohn's Disease over time, with a comparison of two Danish inception cohorts (from 1962-1987 versus 2003-2005) showing a decline in the need for surgery within the first year of diagnosis (35% versus 12%; $P = 0.0001$).[19] Surgical intervention rates differ according to disease phenotype with penetrating disease phenotype having the highest risk of surgery (adj. hazard ratio 5.4; 95% CI 3.0-9.9, $P = 0.001$).[18]

1.1.3 Types of surgical intervention

The most common surgical procedure required in Crohn's disease is the ileo-caecal resection.[16] When the ileo-caecal valve between the small bowel and colon becomes distorted by disease, a bowel obstruction can occur; or in the case of penetrating disease, a fistula can develop leading to the development of an inflammatory phlegmon and/or sepsis.[17] Smaller numbers of patients with isolated small bowel or colonic disease will require small bowel resections or colectomy (partial or total).

Surgical approaches include laparoscopic surgery, conversion surgery (commenced laparoscopically and converted to an open procedure) or a standard open laparotomy. Anastomoses can be either hand sewn or stapled and can vary in configuration (end-to-end, end-to-side and side-to-side), with stapled anastomoses associated with lower rates of complication and possibly disease recurrence.[20, 21]

Other surgical interventions in Crohn's disease include surgery for perianal disease with resection of fistulous tracts and drainage of sepsis.

1.1.4 The Problem of Post-Operative Recurrence

While most patients with Crohn's disease will require surgery at some stage, a proportion of patients require repeated surgery.[22] Often the disease recurs at the site of the previous surgical anastomosis, with 58 - 89% of patients demonstrating post-operative recurrence at 5 years.[11] Endoscopic recurrence is defined as endoscopically identified disease, ulceration, ileitis or anastomotic narrowing, which generally precedes clinical recurrence.[3, 23] Endoscopic recurrence is assessed using the validated Rutgeerts score[3] as shown in Table 1.3.

Score	Appearance of Ileal and Anastomotic Lesions
i0 – Normal	Macroscopically normal anastomosis and ileum
i1	≤5 aphthous lesions
i2 – Recurrence Threshold	>5 aphthous lesions with normal mucosa between the lesions, or skip areas of larger lesions or lesions confined to the ileocolonic anastomosis
i3 – Severe Recurrence	Diffuse aphthous ileitis with diffusely inflamed mucosa
i4 - Severe Recurrence	Diffuse inflammation with larger ulcers, nodules and/or narrowing

Table 1.3 The Rutgeert's Score for Post-operative Crohn's Disease Recurrence.

Adapted from [3]

Clinical recurrence is defined by patient reported symptoms (such as the Crohn's Disease Activity Index – CDAI[24]); however, this is an unreliable measure of endoscopic disease activity, as shown in Figure 1.2. In a pre-anti-TNF study by Olaison *et al* (1992), 93% of patients had anastomotic ulceration, yet only 37% had clinical recurrence.[4] There is a proportion of patients without symptoms who have endoscopic

evidence of disease, making identification of patients at risk of disease progression more difficult to identify. Despite this, the CDAI has been utilised as a marker of clinical recurrence in most post-operative trials, with both a ≥150 and ≥200 threshold.[25] An assessment of the utility of a cut-off CDAI of ≥150 was assessed by the CAST trial, where a 'reasonably good' AUROC of 0.78 was calculated, with a positive predictive value (PPV) of 50% and a negative predictive value (NPV) 91%.[26]

As recurrence is often asymptomatic[27], it may progress silently without (or in spite of) optimal therapy, necessitating structured surveillance to monitor post-operative patients with colonoscopy.[6]

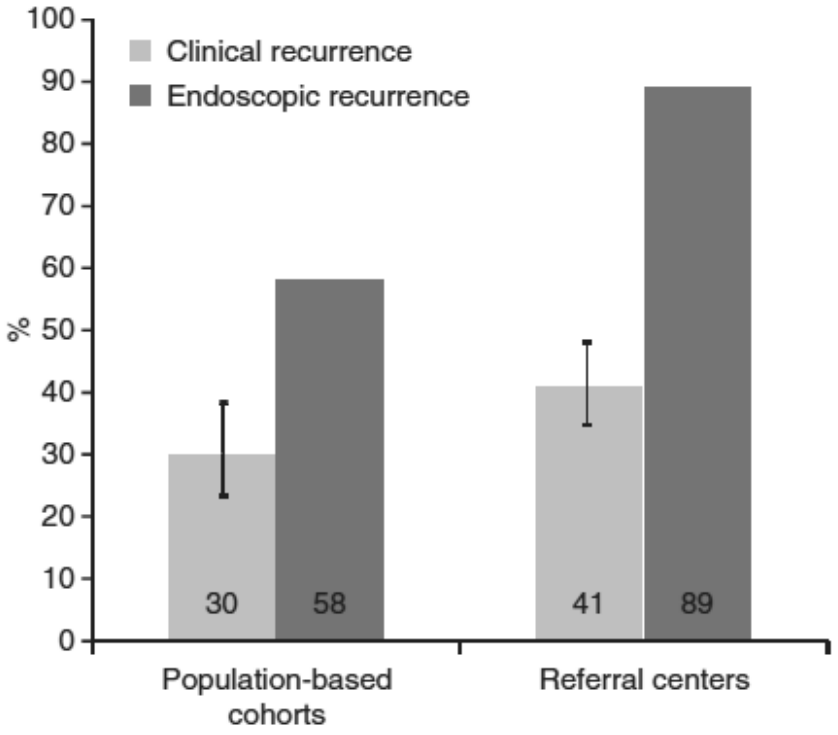


Figure 1.2 Meta-analysis of the rates of clinical and endoscopic disease recurrence after surgery at 5 years, mean values. With permission from [11].

Over time, the "surgery-recurrence-surgery" cycle can be repeated, leading to significant morbidity and loss of bowel length, continuity or function. While rates of disease recurrence are improving with modern treatment, recurrence still represents a significant burden of disease.

1.1.5 The Pathogenesis of Post-Operative Recurrence

Post-operative recurrence is as a result of multiple factors including the microbiome, host immune responses and genetics, disease phenotype, surgical characteristics and contributing environmental risk factors such as smoking.[11, 28-30] These interacting factors all work to contribute to endoscopic and subsequent clinical recurrence (Figure 1.3)

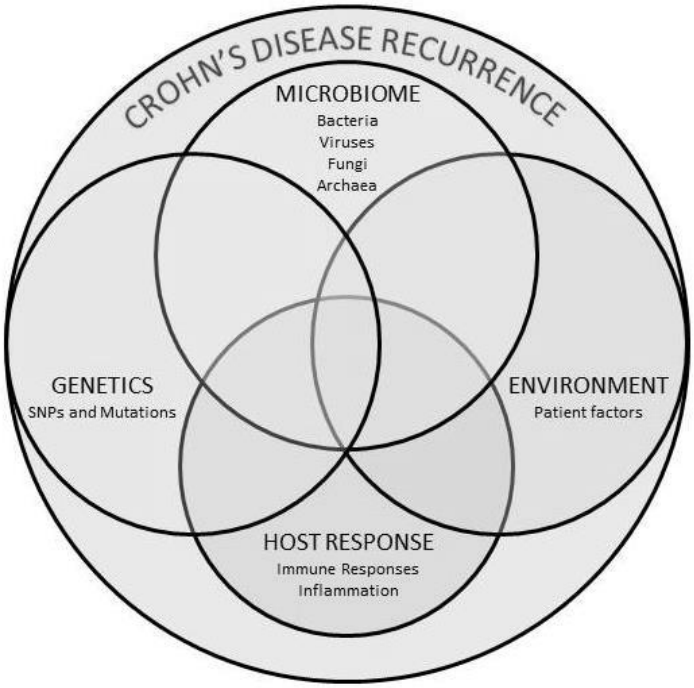


Figure 1.3 Interacting factors involved in the pathogenesis of post-operative recurrence in Crohn's Disease.

1.1.6 The Gut Microbiome and Disease Recurrence

Ileal diversion (creation of a stoma at initial operation) provides some insights into the aetiology of disease recurrence. When a stoma is created, the downstream portion of the bowel is no longer in contact with the faecal stream. Studies following defunctioned patients have shown that the rates of recurrence in the downstream defunctioned bowel is negligible, but disease recurs when the bowel is rejoined.[31, 32] This implies that the faecal stream and the microbiota contained within are required for the development of disease recurrence. Further review of the contribution of the gut microbiome to post-operative disease recurrence is detailed in section 1.5.2.

1.1.7 Host Immune Responses and Genetics

There is some evidence that serologic immune responses may predict patients at elevated risk of disease recurrence but this has not been prospectively tested. This is further reviewed in section 1.3. Myenteric plexitis (neuromatous lesions of the enteric nervous system) at the proximal resection margin have been associated with an increased risk of relapse, with 54% of patients in one series positive for plexitis without surrounding inflammation.[33, 34] When these patients underwent a monitoring colonoscopy three months later, 75% had evidence of recurrence (Rutgeerts score ≥i2, *P* = 0.008).[33]

Li *et al* (2015) looked at the influence of mesenteric lymph node granulomas (from the surgical specimen) on the development of post-operative recurrence. The presence of granulomas in the lymph node was associated with an increase in risk (HR 1.91, 95% CI 1.06-3.45; *P* = 0.031).[35]

Genes such as *NOD2, SMAD3, IGRM* and *CARD8* have been investigated for their contribution to postoperative recurrence risk.[36 29, 37, 38] Mutations in the *NOD2/CARD15* gene have also been investigated to determine their influence on disease recurrence. One study reported at least one *NOD2/CARD15* mutation in 36% of patients undergoing ileo-colic resection, with the presence of these mutations leading to a higher rate of anastomotic recurrence (*P* = 0.01).[39] However, a large systematic review of six cohorts comprising 1003 patients (of which 34% patients had a *NOD2* mutation) showed no increased risk compared to patients with no mutation.[38] *SMAD3* is involved in TGF-β signalling and has been shown to influence both the need for repeat surgery as well as the time to second surgery. In multivariate analysis, *SMAD3* homozygosity was associated with a hazard ratio for recurrence of 4.04 (95% CI 1.77–9.21, *P* = 0.001).[29] *CARD8* (Caspase recruitment domain-containing protein 8) has also been reported to increase the need for recurrent surgery with homozygosity for the risk allele significantly associated with recurrence (OR 7.56, 95% CI 1.13–50.37, *P* = 0.04).[36]

1.1.7.1 Disease Location and other Surgical Characteristics

In a study addressing pre- and post-operative bowel wall thickness (measured by ultrasound), bowel wall thickness that did not improve post-operatively was significantly associated with the risk of repeated surgery when compared to patients where it improved (Hazard Ratio 16.15; 95% CI 2.87–90.75, *P* = 0.002).[40]

A systematic review of anastomosis type in resectional surgery for Crohn's disease has shown that side-to-side stapled anastomoses may be less prone to disease recurrence and complication than a hand-sewn end-to-end anastomosis, with an OR for recurrence of 0.20 (95% CI 0.07-0.45).[21] However, other studies have shown negligible differences between recurrence rates or a benefit from a wider lumen stapled anastomosis.[20, 30]

1.1.8 Risk Factors for Post-Operative Recurrence

A number of risk factors have been reported previously for earlier post-operative recurrence include smoking, previous surgery and penetrating disease.[5]

1.1.8.1 Prior Resection

The risk of endoscopic recurrence is elevated in patients who have undergone a prior resection.[3, 41, 42] In a cohort of 99 patients from St Mark's Hospital London, symptomatic recurrence of disease was more common in patients who had had a previous resection, although this did not reach statistical significance ($P = 0.06$).[41] In a post-operative trial designed to assess the influence of anastomosis type on endoscopic recurrence, a multivariate analysis found that previous resections were associated with both clinical (OR 2.0; 95% CI 1.14-3.60, $P = 0.0016$) and endoscopic recurrence (OR 1.78; 95% CI 1.06-2.90, $P = 0.028$).[42] However, in the POCER cohort, previous resection was not identified as a risk factor for disease recurrence (OR 1.5; $P = 0.41$).[6]

1.1.8.2 Penetrating Disease Phenotype

A meta-analysis of studies addressing the influence of phenotype (defined by the Montreal Classification) on disease recurrence demonstrated that a penetrating disease phenotype (free-perforation, acute or sub-acute perforation and/or fistulisation) was associated with a need for re-operation (HR 1.50, 95% CI 1.16–1.93; $P = 0.002$).[43] While this result was confounded by the heterogeny of the study populations, it additionally demonstrated that patients with surgical indications related to perforations tended to recur with the same phenotype, a trend that has been confirmed in other cohorts.[41, 43] A study addressing the risk of recurrence in 1936 post-operative Crohn's Disease patients demonstrated that the presence of perianal fistulising disease also increases the risk of disease recurrence (OR 1.2; 95% CI 1.03-1.3, $P = 0.013$), as does small bowel and ileocaecal disease location (OR 3.2; 95% CI 2.7-3.6, $P = <0.0001$).[5] In the POCER study, however, there was no link between penetrating disease phenotype

and endoscopic recurrence as measured by the Montreal Classification (B3) (OR 0.9; *P* = 0.78).[6]

1.1.8.3 Smoking

Smoking is one of the best-studied risk factors for recurrence. An analysis of 182 post-operative patients demonstrated an odds ratio of 2.2 (95% CI 1.2-3.8) for recurrence in smokers.[44] A recent meta-analysis of 16 studies looking at the influence of smoking demonstrated a 2.5-fold increased risk of clinical recurrence in smokers compared to non-smokers, and a significant difference in the 10-year reoperation rate.[45] The POCER study also identified smoking as a risk factor for endoscopic disease recurrence (adjusted OR 2.4; 95% CI 1.2-4.8, *P* = 0.02).[6] Smoking has also been shown to induce taxonomic shifts in the gut microbiome, a possible contributor to the pathogenesis of disease recurrence.[46] One of the changes in smokers is an increase in the abundance of *Proteobacteria,* a phylum of gram-negative bacteria that contains a number of pro-inflammatory taxa, such as members of the *Enterobacteriaceae* family.[46]

1.2 Unanswered Questions on the Development of Post-Operative Recurrence

1.2.1 Prediction of Recurrent Disease

The POCER study has helped to define the prospectively identifiable risk factors in patients undergoing surgery. We demonstrated that smoking (OR 2.4; 95% CI 1.2 - 4.8, *P* = 0.02) was an independent risk factor for disease recurrence, as was the presence of two or more risk factors (prior resection, smoking, perforating disease phenotype; OR 2.8; 95% CI 1.01-7.7, *P* = 0.05).[6] This does not identify all patients at risk of recurrence though, and more accurate risk stratification techniques are still required. Interestingly, penetrating disease and prior resection were not statistically significant for recurrence in this cohort; this may be due to short time to follow up (12 months) and the intensification of drug therapy. It is possible that intensive drug therapy with anti-TNF-α drugs ameliorates recurrence more effectively in patients with these risk factors than in patients who smoke, given there is evidence that anti-TNF-α drugs are less effective in smokers.[47] Biomarkers that allow for the prediction of recurrent disease at the time of surgery are currently lacking. Identification of factors that influence risk at the time of surgery would allow immediate post-operative intensification of therapy in order to prevent recurrence in the early post-operative period.

1.2.2 Identification of Recurrent Disease

It is well known that in Crohn's disease patients, active macroscopic disease precedes the development of clinical symptoms.[3, 27] This complicates post-operative surveillance of disease, requiring colonic visualisation of endoscopic disease for optimal management, especially in asymptomatic patients. In one study prior to the biologic era, the rate of endoscopic recurrence at 1 year was 73%; however, the rate of symptom recurrence was 20%.[3]

We have previously advocated for a structured and protocolised follow-up strategy for post-operative patients that includes a surveillance colonoscopy at approximately six months after surgery, regardless of symptoms, to allow for pre-emptive intensification of drug therapy.[6] This reduced the recurrence rate at 18 months post-operatively in the active care (colonoscopy at 6 months) arm to 49% compared to 67% (P = 0.03) in the standard care arm where patients did not intensify treatment.[6]

There is now evidence from the POCER study that faecal Calprotectin is helpful to identify patients with recurrent disease in clinical practice, with a threshold of 100 µg/g having 58% specificity and 89% sensitivity; a negative predictive value (NPV) of 91%.[7]

1.2.3 Treatment and Prevention of Recurrent Disease

1.2.3.1 Medical Therapy for Prevention of Recurrence

A number of pharmacologic strategies have been investigated for post-operative prophylaxis. These include antibiotics, immunosuppressants and anti-inflammatory medications.

Antibiotics

Antibiotics are routinely prescribed following abdominal surgery for prevention of post-operative infection. In the context of Crohn's disease, there is evidence for the effectiveness of longer term (up to 3 months) antibiotics for prevention of recurrence, using antibiotics targeting anaerobic organisims.[23, 48] Rutgeerts *et al* (2005) used 1g per day of ornidazole (a nitroimidazole antibiotic) in a placebo controlled randomised trial for prevention of disease recurrence. At the primary endpoint assessment (at one year post-operatively) the rate of endoscopic recurrence was 79% in the placebo group versus 54% in the ornidazole group as defined by Rutgeert's score ≥i2.[23] A further controlled trial used metronidazole in combination with a thiopurine for prevention and

showed an overall reduction in recurrence when compared to other studies. Although there was no placebo control for the antibiotic administration (all patients received metronidazole for 3 months), the lower rates of recurrence in all patients implies a protective effect of antibiotics.[49] Within the low risk patients (patients with no prior risk factors for disease recurrence) in the POCER study, patients were maintained on metronidazole for the first three months post-operatively, the recurrence rate was 50% versus 70% (P = 0.41) in the high risk patients undergoing intensive drug therapy.[6] This demonstrates that for a select patient population with no identified risk factors, antibiotics may have utility for post-operative prophylaxis.[50]

5-Aminosalicylic Acid (5-ASAs)

In a study of 37 patients with endoscopic recurrence who were treated with thiopurines, the addition of mesalazine (3g/day) had no benefit when added to the drug therapy; in fact, 42% of patients on thiopurine and mesalazine progressed endoscopically, compared to only 19% of control patients (thiopurine only).[51] A further study comparing mesalamine with azathioprine showed no difference in clinical and surgical recurrence patients without previous surgery; however, azathioprine was superior for prevention of clinical recurrence in patients who had had previous resections (OR, 4.83; 95% CI 1.47–15.8, P = 0.03).[52] A Cochrane review of post-operative prevention considers 5-ASAs of modest benefit, although dose and formulation complicate comparisons of these drugs.[53] In clinical practice, for patients with complex disease and/or other risk factors, 5-ASAs are generally not considered sufficient post-operative prophylaxis.[50]

Thiopurines

Thiopurine drugs (purine antimetabolites) including azathioprine and 6-mercaptopurine are commonly prescribed immunosuppressants in Crohn's disease management, including for prevention of post-operative recurrence.[54] Their mechanism of action involves blocking T-cell co-stimulatory signals (CD28) via modulation of Rac1 activation, leading to T-cell apoptosis.[55]

In the post-operative setting, thiopurines have been shown to have moderate efficacy in preventing disease recurrence especially in high risk patients.[50, 52, 56] The effectiveness of thiopurine drugs can be further augmented by the use of combination therapy (often with anti-Tumor Necrosis Factor-alpha antibody drugs). In a head-to-head trial between azathioprine (2mg/kg/day) and mesalamine (3g/day), there was no difference in the clinical recurrence rates as defined by a CDAI score >200.[52] However,

in patients with a history of prior resection (at an elevated risk of recurrence), there was evidence of effectiveness in this population (OR 4.83; 95% CI 1.47- 15.8, *P*= 0.03).[52]

Anti-Tumor Necrosis Factor alpha

Infliximab and adalimumab are two of the most common biologic therapies for Crohn's Disease. Infliximab is a IgG1 murine–human chimeric anti-TNF-α monoclonal antibody[57, 58], whereas adalimumab is a recombinant fully-humanised immunoglobulinG1 (IgG1) anti-TNF-α monoclonal antibody.[59]

A small trial of 58 post-operative patients comparing adalimumab 40 mg fortnightly with standard induction, or azathioprine at the dose of 2mg/kg daily, or mesalamine 3 g/day followed patients for 24 months and assessed disease endoscopically.[60] Endoscopic recurrence rates (assessed using the Rutgeerts score ≥i2) were 6.3, 64.7 and 83.3% respectively (*P* = 0.0017), showing the efficacy of adalimumab in post-operative prevention. Furthermore, the POCER study demonstrated that intensification of therapy at six months with the addition of adalimumab in high risk patients reduced rates of recurrence.[6] A head-to-head comparison of thiopurines versus adalimumab monotherapy in the first six months from surgery also validated a benefit for adalimumab (Recurrence: Rutgeerts score ≥i2; 33/73 (45%) thiopurine versus 6/28 (21%); *P* = 0.028).[61] Clinical guidelines now encourage the early initiation of anti-TNF-α in post-operative patients with risk factors, to prevent progression to endoscopic recurrence.[50]

1.3 Serologic Markers in Post-Operative Recurrence

The gut is protected from direct contact with the microbiome by the presence of a mucous layer. Intestinal dendritic cells sensing commensal bacteria induce the production of secretory immunoglobulin-A (IgA) by plasma cells, with IgA representing >70% of human immunoglobulins.[62] Systemic antibody responses to luminal antigens resulting in production of antibodies of the IgG isotype are also far more common in Crohn's disease patients than healthy subjects.[63]

Patients with Crohn's disease are known to have atypical responses to luminal bacterial flora, resulting in antibody responses to microbial and self-antigens.[64, 65] Antibodies to microbial antigens are common in patients with Crohn's disease and occur as a result of mucosal immune responses to bacterial components such as outer-membrane proteins and flagellin subunits.[66, 67] There is growing evidence of a genetic basis to this loss of tolerance, with elevated anti-microbial antibody levels seen

in first degree relatives of Crohn's Disease patients.[68] This genetic basis may relate specifically to NOD2 mutations that interfere with the intracellular sensing of muramyl dipeptide, a component of both gram-positive and gram-negative organisms.[68-70]

Antibodies to bacterial antigens (such as flagellin proteins) have been associated with disease location, behavior and progression to surgery.[70-72] Antibodies to self-antigens such as pANCA have also been linked with uncomplicated disease and an inflammatory phenotype.[72] These antibodies are usually analysed for both the presence (serum positivity) and magnitude (antibody titre).[66, 71, 72] Measurement of anti-microbial antibodies may assist in clinical practice with diagnosis, prediction of the development of recurrent disease, or with identifying patients at risk of complex disease.[73, 74]

Microbial antigens identified by the presence of antibodies in Inflammatory Bowel Disease patients include oligomannan, cell wall porin proteins and flagellin subunits. Antibodies to mannan cell wall proteins derived from baker's yeast, *Saccharomyces cerevisiae* (ASCA IgG or IgA) are common in Crohn's disease.[75, 76] Omp-C is an outer-membrane porin protein from *E. Coli*, and antigens A4-Fla2 and Fla-X are flagellin subunit proteins that have been phylogenetically linked to *Clostridium* cluster XIVa.[67, 77] While Omp-C antibodies are not routinely measured in Inflammatory Bowel Disease, a recent study has demonstrated that anti-OmpC titers may help predict the responses of Ulcerative colitis patients to anti-TNFα drugs (OR 0.14, 95% CI 0.03–0.6; *P* = 0.04).[78] Antibodies can also develop to "self" antigens, resulting in perinuclear anti-neutrophil cytoplasmic antibodies (pANCA) that have been identified in 70% of patients with ulcerative colitis and in approximately 20% of Crohn's patients. Positivity to pANCA has been associated with a benign disease course in Crohn's disease, but may assist with the diagnosis of Ulcerative Colits.[76, 79]

There is significant variation in serologic reactivity in Crohn's patients from different geographic areas. For example, positivity for ASCA IgG in a Caucasian Crohn's disease population versus Hong Kong Crohn's patients was significantly higher ($P < 0.001$), as was the titre ($P = 0.002$). [80] There are also differences in the rates of positivity due to age, especially in children, with ASCA positivity being rare, but CBir1 being more common.[81] An exploratory investigation of serologic antibodies to predict response to anti-TNF-α showed that there was ASCA positivity was associated with treatment response ($P = 0.0003$).[82]

1.3.1 Serologic Antibodies for Prediction of Complex Disease and Post-Operative Recurrence

There is limited data on the effect of serologic antibody positivity as it relates to post-operative recurrence of Crohn's disease. Previous research has linked the development of some of these antibodies to more aggressive disease,[83] including an elevated risk of surgery[84] and complex disease phenotype.[66] There is some data on the use of serologic antibodies within a predictive model for recurrence which includes other variables such as smoking status and pANCA status, indicating that serologic antibodies alone may be insufficient.[85] A small prospective study assessed the value of ASCA IgG and IgA for prediction of surgical recurrence; however, the lack of variation in the magnitude of ASCA following surgery meant that it was not predictive of recurrence.[86]

Small studies of single markers have demonstrated that serologic antibodies alone may be insufficient to predict disease recurrence in post-operative patients.[74, 85, 87] A study by Eser *et al.* (2012) found that ASCA IgG and IgA were not able to predict endoscopic or surgical disease recurrence.[73] In the present study, we have tested for a wide range of serologic antibodies, providing an opportunity to investigate the prognostic value of a diverse range of antibodies to microbial antigens within a large cohort of well characterised Crohn's disease patients.[28, 88] The impact of the presence and magnitude of these antibodies on the incidence of post-operative recurrence has not been widely assessed. We aim to investigate the impact of these antibodies on the post-operative course of patients enrolled in the prospective POCER study cohort.

1.4 The Gut Microbiome as a Contributor to Crohn's Disease

The gut microbiome is now a known contributor to the aetiology of Crohn's Disease, with the earliest clues coming from the identification of Crohn's disease susceptibility genes involved in bacterial recognition and handling (*NOD2* polymorphisms), implying a role for host-microbiome interactions in pathogenesis.[89] Early microbial community surveys using both molecular and metagenomic techniques demonstrated reductions in the diversity of faecal microbiota (mostly reductions in the Firmicutes phylum) in Crohn's disease patients versus healthy controls.[90-92]

In the last decade, next-generation, high resolution sequencing techniques for gut microbiota have become widely available at a reasonable cost to address the taxonomic shifts within the GI microbial communities to Crohn's Disease.[93] Prior to

these techniques becoming available, investigation of the gut microbiome was limited to culture-based techniques that were unable to fully investigate the fastidious obligate anaerobes that dominate the gut populations.[94] Culture-independent sequencing techniques have allowed high resolution investigations at population rather than species level, revealing the complex interactions of the gut microbiome in both health and disease.[94] Metagenomic surveys of the gut microbiome report both community diversity (alpha and beta diversity), as well as individual taxonomic changes. Alpha diversity relates to the absolute number of species/operational taxonomic units present (a count) within a microbial community, whereas beta diversity (assessed using a metric such as the UniFrac index) assesses community composition between communities (e.g. disease v control, ileum v colon etc.).[95-97]

There are two separate yet related communities within the bowel: the mucosal associated microbiota (MAM) and the faecal associated microbiome (FAM). These community differences relate to the characteristics of the ecologic niche each population occupies, with both longitudinal (stomach→small bowel→colon) and radial (mucosal surface→faecal stream) partitioning.[98, 99] Various patient and environmental factors such as smoking and diet have been shown to modulate microbiome composition in healthy subjects[46, 100] and patients with Crohn's disease.[101]

Broad trends have been identified that differentiate the microbiome of Crohn's disease when compared to healthy controls. These include an overall reduction in alpha diversity in Crohn's disease patients[90, 102], greater overall instability of the microbiome[102], as well as expansion of the *Proteobacteria* phylum (especially the γ-*Proteobacteria*).[103-105] There is also evidence of an overall reduction in Butyrate-producing bacteria from the *Clostridiales* (*Clostridium* clusters XI and XIVa; Table 1.4)[90-92, 106-108]

Clostridium cluster *XIVa*	*Clostridium* cluster *IV*
Clostridium coccoides *Eubacterium (Inc. Eubacterium rectale*) *Ruminococcus* *Coprococcus* *Dorea* *Lachnospira* *Roseburia* (inc. *Roseburia intestinalis*) *Butyrivibrio*	*Clostridium leptum* *Eubacterium* *Ruminococcus* (inc. *Faecalibacterium prausnitzii)* *Anaerofilum*

Table 1.4 Main genera grouped to the *Clostridium* clusters XIVa and IV, containing important Butyrate-producing obligate anerobes.[109-111]

Specific bacterial genera are also perturbed in the microbiome of Crohn's disease patients, including *Faecalibacterium prausnitzii*.[105, 112, 113] *F. prausnitzii* was initially identified as important when mucosal samples from post-operative patients were analysed at the time of surgery and six months later, with patients remaining in remission demonstrating greater abundance.[112] Overall, *F. prausnitzii* can represent up to 5% of the healthy human gut microbiome, and is considered a marker of overall intestinal health.[111, 114] Further analysis demonstrated that *F. prausnitzii* exerted significant anti-inflammatory effects, mainly mediated via blocking of (NF)-κB signalling and down regulation of immune activation by the 15kDa protein MAM.[115]

While there has been increased research on the Crohn's Disease microbiome, limited research has been undertaken to understand the contribution of the gut microbiota to the pathogenesis of post-operative Crohn's disease recurrence, both for single species and the gut microbial communities as a whole.

In order to fully appreciate the contrast between the microbiome of a healthy individual versus a Crohn's disease patient, as well as the changes associated with surgery, the literature on these three populations is reviewed herewith.

1.4.1 The Healthy Human Gut Microbiome

The healthy human gut microbiome comprises of all bacteria, fungi, archaea and viral communities resident in the length of the digestive tract.

Bacteria in particular are classified into ranked groups known as taxa on the basis of differences in morphology, genetics, physiology, metabolism and environment (Table 1.5),[116]

Taxonomic Rank	**Example**
Domain	*Bacteria*
Phylum	*Proteobacteria*
Class	*Gammaproteobacteria*
Order	*Enterobacteriales*
Family	*Enterobacteriaceae*
Genus	*Escherichia*
Species	*coli*

Table 1.5 Example of the taxonomic ranks using *Escherichia coli* [116]

There are six main phyla that comprise the bulk of the gut microbiome: *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria* and the *Verrucomicrobia*, with smaller quantities of other phyla (Figure 1.4).[96, 117, 118] The bacterial population is predominated by members of the *Firmicutes* (mostly members of the class *Clostridia*) and *Bacteroidetes,* comprising up to 90% of the population.[118-121]

The environment of the distal human intestinal tract is a major determinant of the population structure, given it is predominantly devoid of significant oxygen levels.[98] This leads to a majority population of strictly anaerobic and facultatively anaerobic bacteria.[118]

There is a large amount of inter-individual and temporal variation in the gut microbiome, both in terms of the number of species present, and the abundance of the species present.[122 96, 117, 123] Factors that influence an individual's microbiome composition include age, diet, medication and body mass index.[119, 123-125]

A study addressing the unique genes contained in the human gut (using shotgun metagenomics on 124 faecal samples) demonstrated that the average human gut contains approximately 160 species (of which 57 were common to >90% of individuals) derived from a pool of 1000-1150 total species identified across all individuals, although this may be an underestimate.[126] This project also revealed a total of 3.3 million microbial gene sequences across all samples, 150 times more than all identified

human genes demonstrating the huge repository of genetic functionality within the gut metagenome as compared to the size of the human genome.[126]

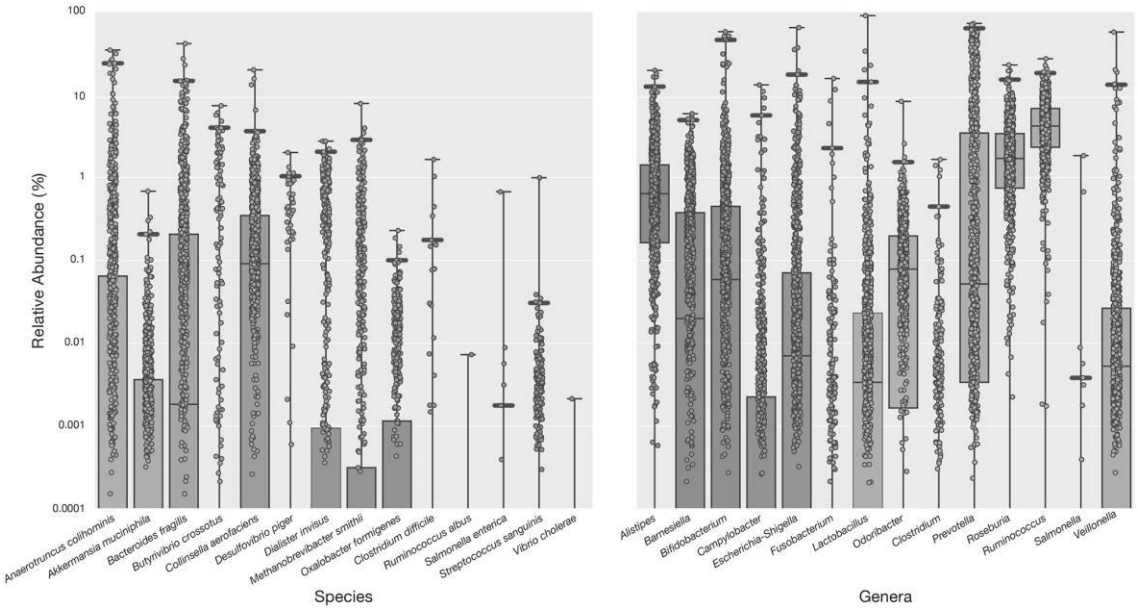


Figure 1.4 Relative abundance of 28 species (left) or genera (right) derived from stool samples obtained from 897 healthy individuals in the first paper to address population reference ranges for taxa from the human gut microbiome. With permission from [122]

1.4.1.1 Longitudinal Partitioning

There are differences in the bacterial communities of the different anatomic portions of the gut, with the small bowel populations differing from the colonic population due to the differences in nutrient availability along the gut (Figure 1.5). The small bowel is rich in amino acids and mono- and disaccharides and supports a bacterial population (particularly *Lactobacilliales* and *Proteobacteria*) adept at metabolising these nutrients.[127, 128]

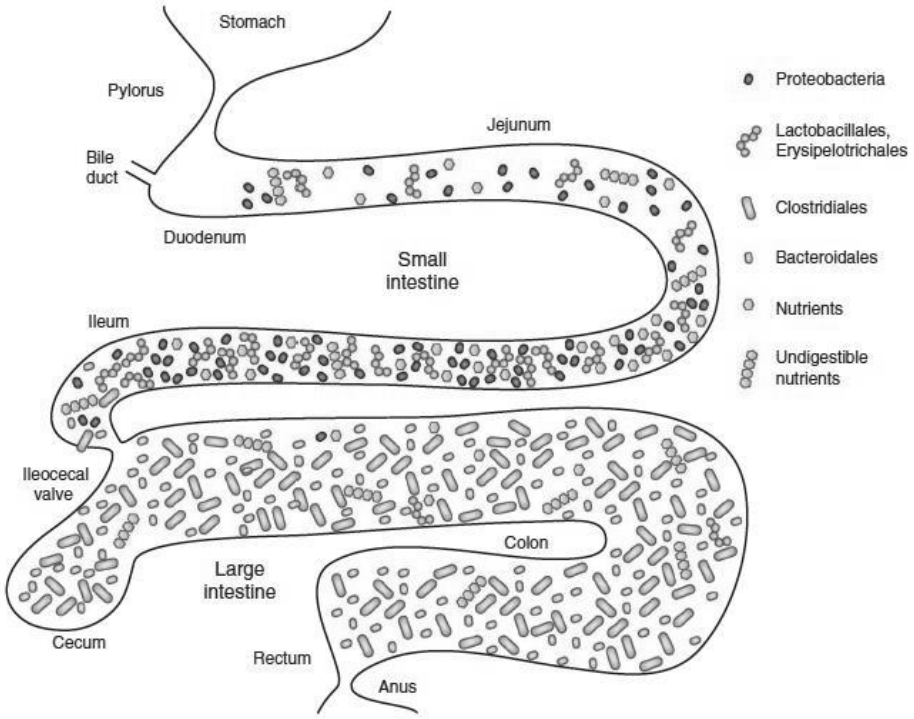


Figure 1.5 Longitudinal populations within the gut by nutrient gradient. The small bowel has increased nutrients relative to the colon, leading to differences in the resident microbiota. With permission from [127]

Oxygen concentration is greater in the proximal small bowel, although this is additionally dependent on host factors.[120] The distal small bowel (around the terminal ileum and ileocaecal valve) is adapted for host-absorption of simple sugars and digestible glycans, which leads to a change in the composition of the flora.[127, 129] The large bowel predominantly metabolises indigestible but fermentable dietary fibre and is an important site of anti-inflammatory butyrate metabolism.[120] The mucosal-rectal microbiome was investigated for oxygen-tolerance, which demonstrated that the aero-tolerant (aerobic, or facultatively anaerobic) bacterial populations are more closely associated with the mucosal surface, possibly related to host-oxygen metabolism. This helps to explain the enrichment of the MAM for Proteobacteria and Actinobacteria.[98] There is also a pH gradient present in the gut, the lowest pH being in the duodenum, small bowel and proximal colon, with the pH gradually rising in the distal colon (Table 1.6).[130, 131]

Stomach	Jejunum/ Proximal Small Bowel	Mid Small Bowel	Terminal Ileum	Proximal Colon	Distal Colon
1.0-2.5	6.63	7.41	7.49	6.37	7.04

Table 1.6 Normal gut pH values for healthy patients. Amended from [130]

1.4.1.2 Radial Partitioning and Oxygen Availability

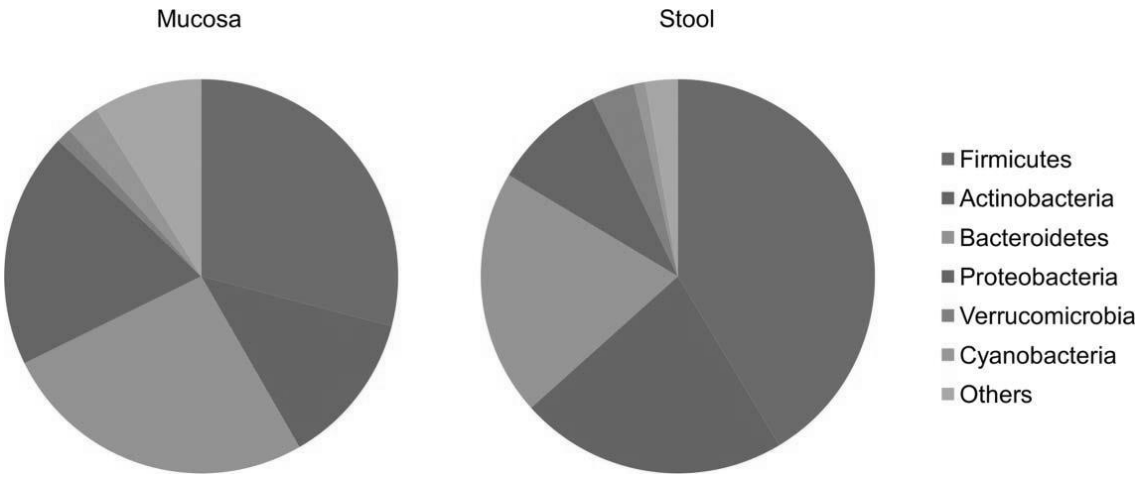
There are distinct compositional differences in the mucosal microbiome and the luminal microbiome, such that they are generally considered to be distinct ecosystems (Figure 1.6).[118, 132, 133] The intestinal environment is for the most part low in available oxygen.[98] There are discernible differences in the oxygen tolerance of the bacteria based on the radial positioning with facultative anaerobes (oxygen-tolerant bacteria) being more closely associated with the mucosa than the obligate anaerobes.[98]

One example of oxygen availability affecting the microbiome is the alteration in the microbiome of patients who have undergone an ileostomy and a small bowel transplant.[134] The creation of a temporary stoma allows a much greater concentration of oxygen to reach the lumen of the small bowel. In these patients, a bloom of oxygen-tolerant, facultative anaerobes was identified, mainly from the *Lactobacilliales* and *Enterobacteriales*, and following closure a rebound of the strict anaerobes such as *Clostridiales and Bacteroidales*.[134]

1.4.1.3 Differences in the Mucosal and Faecal Communities

The mucosal microbiome represents the adherent and oxygen-tolerant bacterial populations, whereas the luminal stream is a composite of shed mucosal bacteria as well as a separate, more oxygen-sensitive, population.[118] The mucous layer of the gut hosts its own unique community, enriched for mucin degrading species such as *Akkermansia muciniphila.*[135] These differences in community diversity and structure complicate efforts to define dysbiosis in the gut, as these taxonomic shifts differ based on the origin of the sample (Figure 1.6).

The mucosal surface also hosts a larger population of adherent and motile bacteria (including from the *Proteobacteria* phylum) that are associated primarily with the mucosal layer of the gut.[120] A study of rectal swab samples versus colonic mucosal biopsies demonstrated differences in the diversity of the communities, with the swabs having a greater diversity (Shannon's Diversity, P= 0.04).[133] This is further reflected in the greater proportion of the *Firmicutes* (such as the anaerobic *Clostridia*) within the luminal stream, and an increase in the facultatively anaerobic genera from the *Proteobacteria* phylum at the mucosal interface.



Phylum	**Stool** (n = 24)	**Colonic Mucosa** (n = 20)	**FDR *P* Value**
Firmicutes	41.4%	29.1%	<0.0001
Bacteroidetes	20.2%	26.3%	<0.05
Actinobacteria	22%	12.6%	<0.0001
Proteobacteria	9.3%	19.3%	<0.0001

Figure 1.6 Relative abundance (proportions) of major bacterial phyla (represented in at least 25% of samples) by sample source. Amended with permission from [132]

Other factors that affect oxygen availability in the gut include pre- and post-operative inflammation altering the availability of terminal electron acceptors such as Nitrate, relative abundance of butyrate producing bacteria and the effect on short chain fatty acid (SCFA) availability. These perturbations are further reviewed in section 1.4.7.

1.4.1.4 Temporal Stability

Interpersonal variability is far higher in children than adults regardless of geographic/environmental factors, with the period up to the age of ~3years of age demonstrating variability and increasing diversity from birth.[123, 136] While the gut microbiome differs significantly from person to person, the diversity and composition of the microbiome is generally stable over time, with 60% of bacterial strains remaining present over a 5 year period.[137] In a study using a phylogenetic microarray specific for the human gastrointestinal tract, the mean intra-individual stability over time was significant (r^2 = 0.96, Pearson correlation).[119] However, significant temporal changes did occur in individuals who received antibiotics or travelled.[119] Age is a strong determinant of the stability of the microbiota, with elderly subjects displaying a breakdown in the normal population structure and an increase in variability.[138]

1.4.1.5 Perturbation of Microbial Metabolism by Antibiotics, Smoking and Other Patient Factors

There are significant differences in the gut microbiome of individuals based on many environmental factors such as ethnicity/geography[123, 139, 140], diet[100, 125], smoking habits[46, 141], antibiotics[142-144], travel[119] and other environmental factors[145-147].

A small study addressing microbiome differences in ethnicity and geography between Australia, Hong Kong and China demonstrated significant differences in bacterial composition.[139] The western lifestyle and diet (when metropolitan US versus rural African versus South American microbiomes are compared) results in significantly less diversity of species (as measured by number of OTUs present; P = <0.005).[123]

There are substantial differences in the gut microbiome over time related to dietary changes.[100, 124] David *et al* (2014) demonstrated drastic shifts in the faecal microbiome based on a plant-based versus animal-based diet. The animal-based diet (when subjects were switched from a plant-based diet) cause the greatest (and fastest) shift in composition, including a significant reduction in *Prevotella*, an important genus in fibre metabolism, with changes occurring within one day of the dietary changes. Furthermore, there was an increase in bile-acid production and in the abundance of *Bilophila wadsworthia* in those subjects that consumed an animal-based diet.[100] *Bilophila wadsworthia* is a hydrogen-sulfide producing bacterium that has been shown to perpetuate inflammation.[100]

Smoking has an impact on the structure and diversity of the bacterial communities in the gut, with differences in composition (between smokers and non-smokers) that can range from mild[124], to more distinct in Inflammatory Bowel Disease patients.[148] Studies addressing changes associated with smoking cessation demonstrated that populations of *Firmicutes* and *Actinobacteria* increase and *Bacteroidetes* and *Proteobacteria* decrease in abundance.[46] Overall, there was a significant increase in α-diversity on cessation of smoking, at least in the short term (4 weeks).[46]

Research addressing the effect of broad-spectrum antibiotic consumption has shown these drugs cause large taxonomic shifts in the gut. A cohort of 21 patients was treated with fluroquinones and β-lactams, and their faecal microbiome was analysed. This revealed a decrease in the phylogenetic core from 29 to 12 taxa and a decrease in *Firmicutes* abundance with a concomitant rise in *Bacteroides*.[149] Overall, administration of these antibiotics significantly reduces microbial diversity, and recovery to the pre-antibiotic state requires time and may be incomplete.[149, 150] A study addressing the impact of a *Helicobacter pylori* eradication regime involving both Clarithromycin and Metronidazole (with omeprazole) demonstrated significant long term (up to four years) shifts in the faecal microbiome, including an overall decrease in diversity that improved over time.[142]

Other environmental factors that may influence the gut microbiome include co-habitation and pet ownership.[146, 151]

1.4.2 The Single Pathogen Hypothesis of Crohn's Disease versus a Microbial Ecology View

There are a number of single species that are associated with Crohn's Disease and/or disease recurrence. These include *Escherichia coli*[152], *Proteus spp.*[153, 154], *Campylobacter spp.*[155], *Fusobacterium spp.*[156], *Serratia spp.*[157], *Faecalibacterium prausnitzii*[112] and *Mycobacterium paratuberculosis*[158, 159]. However, the literature is far from conclusive on the contribution of these species to the disease process.

The "single pathogen" hypothesis for the aetiology of Crohn's disease has been investigated for a number of candidate microbial agents, such as *Mycobacterium avium* subsp. *paratuberculosis* and adherent-invasive *Escherichia coli* (AIEC).[160] A randomised trial on antibiotic treatment for *M. avium* subsp. *paratuberculosis* was not found associated with reduction of Crohn's disease.[160] *E. coli* is a high abundance commensal organism, and while it may acquire additional virulence factors such as

Type I pili that allow adherence to mucosal surfaces (AIEC), a causal relationship to the development of Crohn's disease has not been proven.[160]

One theory is that a change in the gut environment, by any of the factors mentioned above can cause an ecologic change in the microbiome by selecting for microbes tolerant to the perturbation. This can lead to an expansion of "pathobionts", defined by Chow, Tang and Mazimanian (2011) as "resident microbes with pathogenic potential". These include the species listed in Table 1.7. This alteration in the luminal microenvironment includes the liberation of amino acids as a result of tissue destruction, which favours the expansion of auxotrophic bacteria.[148] Auxotrophy is defined as "the requirement for the external supply of a nutrient", such as amino acids, nucleotides or other metabolites that are required the metabolism of the cell or organism.[148, 161] This shift from a normal metabolic profile (basic biosynthesis) to an auxotrophic profile that favours populations of metabolically flexible pathobionts such as the aerotolerant *Proteobacteria* has been identified in a number of studies of inflammatory bowel disease patients.[105, 148]

Bacterial Strain	Potential Pathogenesis	Reference
Proteus species§	HpmA haemolysis from *Proteus mirabilis* may induce inflammation by inducing IL-1β (via the NLRP3 inflammasome (further reviewed in chapter 5)	162, 163
	Microarray of faecal samples from healthy controls and ileal Crohn's disease patients showed increases in *Proteus vulgaris* in the microbiome of Crohn's disease cases.	157, 164
	Interactions between a number of species from the *Enterobacteriaceae* family and the mycobiome may perpetuate formation of a pro-inflammatory biofilm (Positive correlation between presence of *Proteus* genus and Candida, $r^2 = 0.709$; $P = 0.005$).	
	A mouse model of ulcerative colitis (TRUC mouse, a *T-bet*$^{-/-}$ x *Rag2*$^{-/-}$ knockout model) was used to demonstrate that *Proteus* sp. and *Klebsiella* sp. can elicit colitis in TRUC mice, and that this dysbiosis can be transmitted to wild type mice via microbiome transfer.	
Klebsiella pneumoniae§		162
	A high starch diet allows population expansion of *Klebsiella.* This the perpetuates immune reactivity and auto-antibody production against mucosal collagen following exposure to *Klebsiella* pullulanase enzyme (pulA), which shares epitopes with collagens I, II, and IV present in the bowel.	165
Serratia marcasens§	Abundance positively correlates with the abundance of *Candida tropicalis* in Crohn's Disease cases. Potential for fungal/bacterial interactions to produce a robust biofilm of hyphae/bacterial flagella and lipopolysaccharides that may perturb intestinal homeostasis.	157

Campylobacter concisus	Identified in intestinal biopsy specimens from children newly diagnosed with Crohn's Disease at higher levels than in controls, and correlated with increases in IgG antibodies to *C. concisus* in the serum of the cases.	[155]
Fusobacterium nucleatum	Present in the normal gut flora of 23% of sampled children, as compared to 82% of children with Crohn's disease.	[155]
Escherichia coli[§] (Adherent-invasive type – AIEC)	Has been recovered from mucosa of Inflammatory Bowel Disease patients (64%), as compared to healthy patients (27%; $P = 0.01$) undergoing cancer screening. Strains recovered from Crohn's disease patients with inflammation were more invasive than those from controls ($P < 0.001$ as measured by Caco-2 cellular invasion assays). Surveys of AIEC in healthy control and Crohn's disease biopsy specimens have demonstrated higher prevalence, greater abundance and deeper species richness in Crohn's patients.	[156] [152]

Table 1.7 Previously identified pathobionts of the human gut involved in Crohn's Disease. Species listed with [§] are members of the *Enterobacteriaceae* family.

1.4.3 The Mucosal Microbiome in Crohn's Disease

Most research on the microbiome of Crohn's disease has focused on the mucosally associated microbiome, as Crohn's is a mucosal and transmural condition, and mucosal biopsies can be obtained from the site of active disease.

When both mucosal and faecal samples from Crohn's disease patients were compared with controls, the directionality of change in the microbiome was the same in all samples, but the magnitude differed.[105] The mucosal samples were enriched for *Enterobacteriaceae*, *Pasteurellaceae*, *Fusobacteriaceae*, *Neisseriaceae*, *Veillonellaceae* and *Gemellaceae*. Reductions in the orders *Bacteroidales* and *Clostridiales*, as well as the *Erysipelotrichaceae* and *Bifidobacteriaceae* were also demonstrated.[105] However, this dysbiosis was not reflected in the faecal samples, complicating the non-invasive investigation of Crohn's disease.[105]

Aerotolerant organisms such as those from the *Proteobacteria* phylum and specifically from the genus *Enterobacteriaceae* (such as *E. coli*) are known to be more associated with the mucosal surface and may undergo population expansion during gut inflammation, not least due to the increase in oxidative metabolites produced during infection.[98, 166, 167] Adherent invasive strains of *E. coli* have been associated with Crohn's disease, and are known to be predominantly mucosally adherent. [152]

1.4.4 Post-operative Mucosal Microbial Dynamics

An early study using culture-based techniques looked at the dynamics of the bacterial population in Crohn's disease patients and controls after ileal resection, demonstrating an increase in bacterial counts (and of *E. coli* and enterococci) following surgery in Crohn's disease patients.[168]

Sokol and colleagues (2008) demonstrated that a lower abundance of *Faecalibacterium prausnitzii* in the ileal mucosa at surgery was associated with an elevated risk of recurrence in Crohn's disease patients and that this alteration remained six months post-resection.[112]

A small study of the mucosal microbiota in six post-operative Crohn's patients (at time of surgery and post-operatively) showed taxonomic shifts including reduced abundance of *Lachnospiraceae*, *Erysipelotrichales* and *Firmicutes* and increased abundance of *Rhodobacteraceae* and an unknown *Proteobacteriaceae* at time of surgery in patients

with recurrence.[169] An overall reduction in microbial diversity was also present in patients with recurrence when compared with healthy controls.[169]

A recent study by Mondot et al (2016) showed changes present pre-operatively in Crohn's disease patients undergoing surgery including enrichment for α/β *Proteobacteria*, and *Streptococcus*. At six months post-operatively, there was an increase in mucosally-associated *Clostridia*, mainly *Lachnospiraceae* (*Dorea* and *Blautia*). Overall, there were 27 differentially abundant mucosally-associated OTUs between baseline and 6 months but no differences in α-diversity (Shannon index, Baseline 2.538 ± 0.3179 versus 6 months 3.030 ± 0.111, P >0.05).[153] *Porphyromonadaceae* was enriched in six month samples from patients with disease recurrence.[153]

A pilot analysis of the mucosal microbiota in 12 POCER patients at baseline and at 6 months post-operatively (using microarray and 454 pyrosequencing) demonstrated a lower abundance of *Bacteroidetes* in patients with recurrence, as well as an overall reduction in bacterial diversity.[170] In baseline samples from patients with recurrence at 6 months, there was a decrease in the *Clostridiales* and *Bacteriodales* (excluding Blautia), and an increase in the *Streptococcaceae* and *Enterococcaceae* (from the Bacilli order), as well as *Veillonellaceae* and *Enterobacteriaceae*. [170]

Larger scale longitudinal analysis of the POCER cohort (141 samples from 34 patients) by Wright et al (2016) showed lower phylogenetic ("alpha") diversity (number of different species found) in Crohn's patients versus healthy controls, but no differences between time points or remission and recurrence. When the relative abundance of species was assessed (beta diversity), differences were seen between baseline resection and 6 and 18-month samples. There were no significant differences in beta diversity between patients with disease recurrence or remission at 6 or 18 months. When specific taxa were assessed, there was a reduction in abundance of phylum *Bacteroidetes* as seen in previous work[170], and altered abundance of a further 11 families (including ↓*Lachnospiraceae*, ↑*Clostridiaceae*, ↑*Pseudomonaceae*, and ↑*Lactobacillaceae*) and 14 genera (including ↑*Pseudomonas*, ↑*Clostridium*, ↑*Anerostipes*, ↑*Lactobacillus*, ↑ *Epulopiscium* and ↑*Turicibacter*) when baseline samples were compared with 6 months. Disease recurrence at 6 months was associated with the presence of *Proteus* spp. (P = 0.008); logistic regression analysis (corrected for smoking status) demonstrated that compared to patients with no detectable *Proteus* spp. the odds ratio for recurrence was 13.0 (1.1-150), P = 0.039. A

low abundance of the anti-inflammatory species *Faecalibacterium prausnitzii*, when combined with a patient's smoking status and presence of *Proteus* spp. was shown to have moderate accuracy (AUC 0.740) for prediction of recurrence.[154]

1.4.5 The Faecal Microbiome in Crohn's Disease

The luminal (faecal) microbiome of Crohn's disease is enriched for obligate anaerobes when compared to the mucosal surface.

An overview of the increased and decreased taxa associated with Crohn's Disease when compared to healthy patients is given in Table 1.8.

Study	N Patients	Increases in	CD Decreases in	Technique
Pascal, 2017	67 IBD patients (CD and UC) 111 Healthy Controls	*Fusobacterium* *Escherichia*	*Faecalibacterium* An unknown *Peptostreptococcaceae* *Anaerostipes* *Methanobrevibacter* An unknown *Christensenellaceae* *Collinsella*	16S
Ijaz, 2017	17 Unaffected Relatives 14 Healthy Controls 19 CD Children	*Enterobacteriaceae, Pasteurellaceae* *Veillonella* *Dorea* *Anaerostipes* *Enterococcus* *Clostridium_XVIII* *Clostridium XIVa* Subcluster	*Ruminococcaceae* *Lachnospiraceae* *Phascolarctobacterium* *Parabacteroides* *Akkermansia* *Methanobrevibacter*	16S
Maukonen, 2015	10 CD Children 12 UC Children 8 Healthy Children	-	*Lachnospiraceae* *Coriobacteriaceae* *Bifidobacterium* *Ruminococcaceae* (diversity reduction)	PCR (DDGE)
Kohlo, 2015	68 IBD Children 26 Healthy Controls	*Bacteroides* *Clostridium* *Eubacterium*	*Faecalibacterium*	Microarray qPCR

Study	N Patients	Increases in	CD Decreases in	Technique
Hedin, 2015	22 CD patients 21 CD siblings 25 Healthy Controls	*Escherichia coli*	*Faecalibacterium prausnitzii* *Clostridia* cluster IV *Ruminococcus spp.* *Roseburia spp.* *Bacteroides-Prevotella* *Bifidobacterium adolescentis*	16S qPCR
Gevers, 2014	477 CD patients (faecal samples from 223 patients only) 221 Control patients (Shotgun metagenomics performed on 33 CD patients and 10 controls)	*Streptococcus*	*Lachnospiraceae* (including *Dorea, Ruminococcus* spp. and *Blautia*)	16S Shotgun metagenomics
		Escherichia coli *Veillonella parvula* *Eikenella corrodens* *Gemella moribillum.* *Fusobacterium nucleatum* *Haemophilus parainfluenzae*	*Bacteroides vulgatus* and *caccae,* Multiple species of *Bifidobacterium* *Blautia hansenii* Multiple species of *Ruminococcus* Multiple species of *Clostridium* *Faecalibacterium prausnitzii,* *Eubacterium rectale* *Roseburia intestinalis* *Coprococcus comes*	Shotgun metagenomics
Joossens, 2011	68 CD 84 Unaffected Relatives 55 matched Healthy Controls	*Ruminococcus gnavus*	*Dialister invissus* Unknown *Clostridium* cluster XIVa *Faecalibacterium prausnitzii* *Bifidobacterium adolescentis*	DDGE

Study	N Patients	Increases in	CD Decreases in	Technique
Kang, 2011	6 CD patients 6 Healthy Controls	*Enterococcus* *Clostridium difficile* *Escherichia coli* *Shigella flexneri* *Listeria*	*Eubacterium rectale* *Bacteroides fragilis* group *Bacteroides vulgatus* *Ruminococcus* spp. *Faecalibacterium prausnitzii*	Microarray qPCR
Andoh, 2009	34 CD Patients 30 Healthy Controls	*Bacteroides* *Enterobacteriales*	*Clostridium* Clusters IV and XI *Clostridium* Subcluster XIVa	T-RFLP

Table 1.8 A review of faecal taxonomic results comparing healthy control samples with faecal samples from Crohn's Disease patients. Abbreviations: 16S – Sequencing of the 16S small ribosomal subunit gene, PCR – Polymerase Chain Reaction, DDGE – Denaturing Gradient Gel Electrophoresis, qPCR – Quantitative Polymerase Chain Reaction, T-RFLP - Terminal restriction fragment length polymorphism, CD – Crohn's Disease, UC – Ulcerative Colitis

1.4.5.1 Post-operative Faecal Microbial Dynamics

Mondot et al (2016) addressed changes in the faecal microbiome of patients with matched mucosal samples (see section 1.4.4). Using Temporal Temperature Gradient Gel Electrophoresis (TTGE) to address similarity (analysed by Pearson correlation method with unweighted pair group method with arithmetic mean) between populations and timepoints, they described variability between baseline and 6 month samples (similarity percentage 41.3% ± 34.1%), as well as differences between patients in remission versus those with recurrence at 6 months (similarity percentage; Remission 45.1% ± 23.4% v Recurrence 58.1% ± 30.1%).[153]

Halfvarson (2017) addressed the dynamics of the faecal microbiome in 25 post-operative Ileal Crohn's patients versus six patients with ileal Crohn's disease and no history of surgery. The patients who had resections demonstrated greatest volatility between subsequent samples (assessed using UniFrac distances), as well as lower species richness.[171] When differential abundance between post-operative and non-operative patients was assessed, significant fold change was seen for *Faecalibacterium prausnitzii*, the *Lachnospiraceae*, four taxa from the *Ruminococcaceae* family, as well as the *Ruminococcus* genus and the *Clostridiales* order.[171]

In a small sample of patients (n = 54) who provided faecal samples prior to and six months after ileo-caecal resection, patients who developed recurrence (Rutgeerts i3-4) had an elevated abundance of *Streptococcus* ($P = 0.002$); however, this was not significant after correction for false discovery rate.[102]

1.4.6 Smoking, Antibiotics and Environmental Factors – the Effect on the Microbiome of Crohn's Disease Patients

1.4.6.1 Smoking

In Crohn's patients, smoking is associated with increased abundance of *Bacteroides-Prevotella* families and has been independently associated with increased rates of post-operative recurrence.[6] Morgan *et al* (2012) demonstrated a decrease in *Anaerostipes* (*Lachnospiraceae* family, from the *Firmicutes* phylum) of up to 60% abundance when current and past smokers were compared to non-smokers. This

genera is considered a contributor to gut health as it is a lactate-utilising butyrate producer.[172]

1.4.6.2 Antibiotics

There is increasing evidence that antibiotics may perturb the overall ecology of the gut, both in Crohn's patients and healthy controls. The gut microbiome is ruled by the principle of "competitive inhibition", where relative abundance of bacteria taxa is controlled by availability of resources (SCFA, electron acceptors such as nitrate for anaerobic respiration and O_2).[173-175] When antibiotics remove keystone groups such as butyrate-producing taxa (*Clostridia* and other obligate anaerobes), there is a change in the availability of resources on which other taxa depend.[174] For instance, use of the antibiotic streptomycin in mice leads to an expansion of *Salmonella enterica* serovar Typhimurium, mediated by an increase in inducible nitric oxide synthase (iNOS).[176] This increase in reactive nitrogen species increases the colonic abundance of galactarate and glucatate, which are carbon sources for catabolism, providing a growth advantage to *Enterobacteriaceae* (such as *Salmonella* and *E. coli*) that can utilise that energy pathway.[176]

Furthermore, coloncyte metabolism (β-oxidation of butyrate to CO_2 by colonocyte mitochondria) is important in maintaining a hypoxic environment in the gut as it consumes O_2.[177] When the butyrate producers are removed by antibiotics and the metabolic substrate concentration (butyrate) is reduced, colonocytes move to ferment glucose into lactate, a process that increases the luminal oxygen concentration.[177] This in turn favours expansion of facultative anaerobes such as the *Enterobacteriaceae,* from the *Proteobacteria* phylum (Figure 1.7).[175]

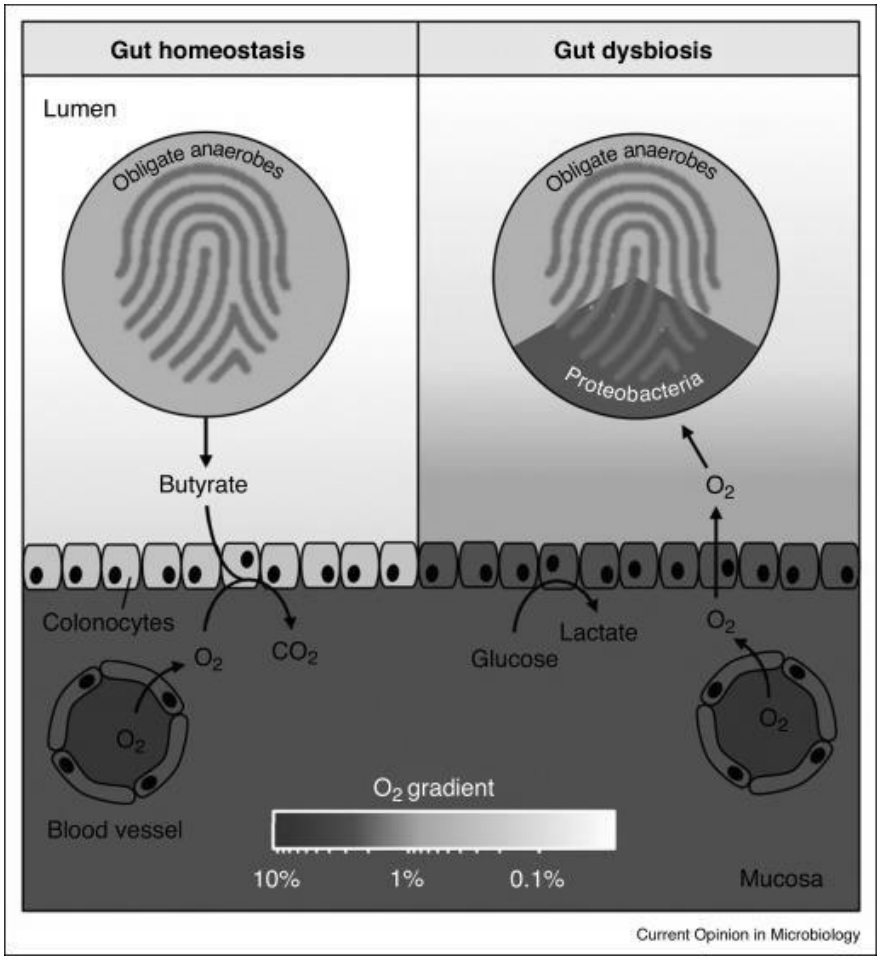


Figure 1.7 Oxygen concentration as a driver of Proteobacterial expansion after antibiotic administration. Left, normal colonocyte metabolism of butyrate. Right, perturbation of oxygen concentration by a switch of colonocyte metabolism from oxidation of butyrate to fermentation of glucose. With permission from [178]

1.4.7 The Ecologic Consequences of Inflammation

The presence of inflammation (either through the disease process in Crohn's disease or via surgery) can have a profound effect on the gut microbiome. Inflammation increases the availability of terminal electron acceptors for microbial facultative anaerobic metabolism by the *Enterobacteriaceae*, such as Nitrate, Nitrite, S-oxides (tetrathionate), N-oxides and fumarate.[173, 179, 180] Breakdown of colonocytes provides further alternative respiratory electron acceptors via ethanolamine obtained from phospholipid/cell-wall breakdown[181], as well as trimethylamine derived from phosphotidylcholine.[182, 183] Additionally, neutrophil recruitment, degranulation and respiratory burst in inflamed tissues releases reactive oxygen species (ROS) that react

with endogenous sulphur containing compounds to produce tetrathionate, an electron acceptor for *Salmonella* and *Proteus* spp.[116, 180]

The wide range of anaerobic respiration pathways available to the *Enterobacteriaceae* in the inflamed bowel provides a significant advantage to these bacteria, promoting population expansion in the inflamed bowel.[182]

1.5 The *Enterobacteriaceae* Family and the Proteus Genus in Health and Inflammatory Bowel Diseases

1.5.1 The *Enterobacteriaceae* Family

Members of the *Enterobacteriaceae* have been linked to Crohn's Disease as early as the 1970s using culture based techniques.[184] Further, more recent observations demonstrated that patients with ileal Crohn's Disease have elevated counts of *E. coli* even when still using culture-based methods.[168] The move to molecular-based analysis has further strengthened the link, with many groups identifying Proteobacterial expansion[103-105], specifically the *Enterobacteriaceae* as an identifying characteristic of Crohn's Disease dysbiosis.

1.5.1.1 Characteristics of the Family

The *Enterobacteriaceae* are common, low abundance (<0.1% total) residents of the normal human gastrointestinal tract.[118] The phylogeny of the *Enterobacteriaceae* is shown in Figure 1.8. This family represents one of the most prevalent and diverse contributors to the gastrointestinal microbiome.[185] The *Enterobacteriaceae* include a number of well-known pathobionts known to cause disease in certain circumstances. These include food borne pathogens (*Salmonella enterica, Shigella dysenteriae, Yersinia enterocolitica, E. coli*), the causal agents of typhoid and bubonic plague (*Salmonella typhi* and *Yersinia pestis*) and many bacteria known to cause secondary and nosocomial infections (*Proteus mirabilis, Klebsiella pneumonia, Serratia* spp., *Providencia* spp., *Morganella* spp., *Enterobacter* spp.).[116] As gram-negative bacteria, they possess pro-inflammatory lipopolysaccharide (LPS) as part of their cell wall, which can cause a severe host inflammatory response.[186] Many *Enterobacteriaceae* are also motile (produce flagella) and adhesive (produce fimbriae).[116, 187]

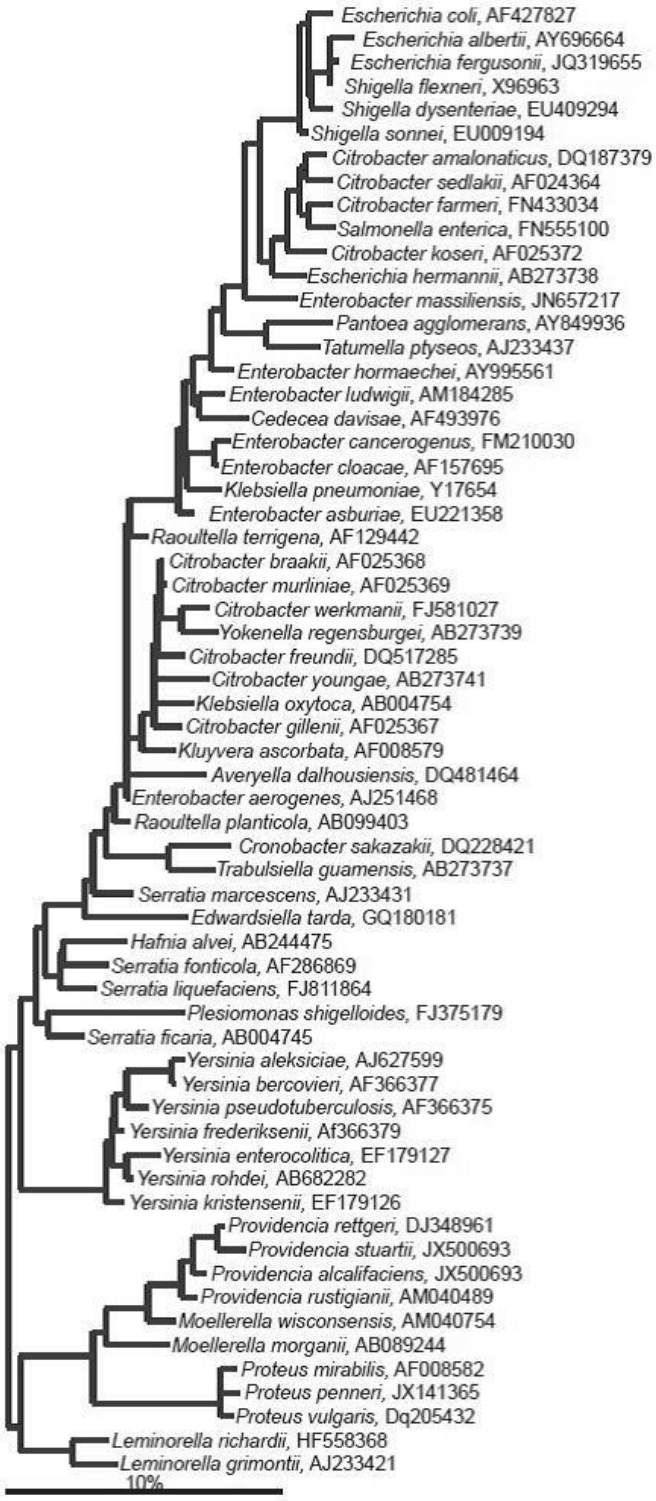


Figure 1.8 Phylogenetic tree showing the members of the *Enterobacteriaceae* based on the 16S phylogeny. The black bar shows percentage divergence of the 16S gene between species. With permission from [185]

1.5.1.2 Metabolic Pathways

The *Enterobacteriaceae* are hardy and adaptable to their environment. As chemoorganotrophs they can utilise both aerobic and anaerobic respiration.[116] This may account for their ubiquity in the environment and in the human microbiome. The *Enterobacteriaceae* can use a wide range of terminal electron acceptors for anaerobic respiration, allowing growth in a wide range of ecologic niches while outcompeting more fastidious and obligately anaerobic species.[188]

1.5.2 The *Proteus* Genus

Proteus species are motile, gram-negative pathogenic bacteria, first described in 1885 by Gustav Hauser.[189, 190] The dimorphic nature of *Proteus* species is referenced in the genus name, with *Proteus* able to transform from the single-cell "swimmer" morphology to highly flagellated, multinucleated "swarming" forms.[191] Hauser took inspiration from Homer's Odyssey; the name "Proteus" is a reference to Proteus, the Greek god of rivers and oceans who evaded capture by transforming himself into many forms.[192]

Proteus species are best known as urinary tract pathogens, being a common cause of urolithiasis, prostatitis, pyelonephritis and catheter-associated urinary tract infections.[190] The relationship of the *Proteus* genus to other members of the *Enterobacteriaceae* is shown in Figure 1.8.

1.5.2.1 Characteristics of the Genus

Relevant characteristics of the genus *Proteus* are shown in Table 1.9.

Phylum Class Order Family Genus Species[193]	*Proteobacteria* *Gammaproteobacteria* *Enterobacteriales* *Enterobacteriaceae* *Proteus* *Proteus mirabilis, Proteus vulgaris, Proteus penneri, Proteus hauseri*
Characteristics[190]	Gram-negative Dimophic Facultatively anerobic[194] Swarming motility
Clinical Significance[190] (*P. mirabilis)*	Can cause pyelonephritis, urolithiasis, and prostatitis. Leading cause of nosocomial urinary catheter infections. *Proteus* in UT is acquired from GI tract → self-inoculation. Has also been linked to diarrheal illness.[195]
Virulence Factors (*P. mirabilis)*	**Urease positive** – increases the availability of nitrogen for microbial metabolism [190] **Fimbrae** – 17 putative fimbral operons have been identified in the *P. mirabilis* genome.[196] ↑ in ability to adhere to epithelial cells of UT and possibly GI tissues, increasing invasivness[190] **Flagellated** – *P. mirabilis* displays swarming motility in semi-solid and solid media,[190] potentially a virulence factor for GI luminal colonisation. **Toxin Production** – *P. mirabilis* and *vulgaris* produce haemolysin, which is cytotoxic to human renal proximal tubular epithelial cells.[197] Secretion of this toxin may contribute to any pathogenic effects in the GI tract. **Plasmid acquisition** – One fully sequenced strain (HI4320) possesses a extrachromosomal plasmid encoding a full set of genes for conjugal transfer that may increase the ability of this strain to horizontally acquire or transfer virulence factors such as antibiotic resistance casettes.[196] **Immune Evasion** – Some *Proteus* species (including *P. mirabilis*) produce metalloprotease ZapA, which cleaves immunoglobulins IgA1, IgA2 and IgG, preventing opsonisation and microbial clearance.[190] ZapA can hydrolyse cell matrix proteins including collagen, laminin and fibronectin, as well as complement proteins C1q and C3.[198]

Table 1.9 Relevant Characteristics of *Proteus* species. Abbreviations - UT – Urinary Tract, GI – Gastrointestinal

1.5.2.2 Proteus in Gastrointestinal Disease

Analysis of the 16S sequencing of intestinal biopsies from the POCER cohort has shown a strong preliminary link between the mucosal presence of *Proteus* spp. and disease recurrence at 6 months (OR 13 (1.1-150), *P* = 0.039) when corrected for smoking.[154]

There are a small amount of data that suggest the presence and/or pathogenicity of *Proteus* spp. in Crohn's disease may have a role in disease. *Proteus* spp. have been recovered from the faecal stream of both healthy patients and patients with gastrointestinal symptoms and from the serosal surface of Crohn's patients during surgery.[195, 199] *Proteus* spp. have also been shown to be an occasional contaminant of endoscopic equipment, which may play a role in gastrointestinal transmission.[200]

Proteus species (especially *P. mirabilis*) are finely adapted for invasion and infection of mucosal surfaces and evasion of host immune responses. It is plausible that due to the multitude of virulence factors possessed by *P. mirabilis*, this species may cause disease at low abundance within the gut microbiome. Low abundance species such as *Salmonella* can still cause clinically significant disease at less than 0.01% of the gut microbiome, although this is often accompanied by a population expansion during clinical illness.[122] Lack of techniques for fine resolution of microbial community composition prior to the adoption of next-generation sequencing techniques may explain the absence of literature on *Proteus mirabilis* as a potential pathogen in the gastrointestinal tract. This may be further compounded by the adoption of sequence abundance thresholds (>1%) in 16S metagenomic analyses, where OTUs comprising less than 1% of total filtered reads are discarded. Further research on *Proteus* species as a contributor to gastrointestinal disease, especially inflammatory bowel diseases, is required.

1.6 Scope of Research in this Thesis

1.6.1 Overall Hypotheses of this Research

1.6.1.1 Microbial Serology in Post-Operative Crohn's Disease

- Serologic antibodies correlate with phenotypic characteristics at baseline in the Australian population
- The presence and/or magnitude of serologic antibodies is associated with the development of endoscopic disease recurrence

- The presence and/or magnitude of serologic antibodies can predict the development of endoscopic disease recurrence
- Specific serologic antibodies may be associated with elevated faecal calprotectin (> 100 µg/g) following intestinal resection.

1.6.1.2 The Post-Operative Faecal Microbiome in Crohn's Disease

- There are differences in the microbial community composition between:
  - Patients with disease recurrence at 6 and/or 18 months versus those patients who remain in remission
- There is a faecal microbial profile that is associated with the occurrence of (or protection against) endoscopic recurrence, and this can be identified at the time of resection
- *Proteus* spp. are present in the faecal-associated microbiota of patients with disease recurrence

1.6.1.3 Proteus species in Gastrointestinal Disease

- *Proteus* species are adapted to the gastrointestinal tract and may be an unusual and under-appreciated cause of gastrointestinal diseases
- The presence of *Proteus* spp. in the gastrointestinal tract is associated with the development of Inflammatory bowel diseases
- The innate and acquired virulence factors of the *Proteus* genus may contribute to the pathogenesis of gastrointestinal disease
- That *Proteus* spp. (and other members of the *Enterobacteriaceae*) may play an important pathogenic role at the mucosal surface in patients with inflammatory bowel diseases, specifically Crohn's Disease.

1.6.2 Overall Aims of this Research

1.6.2.1 Microbial Serology in Post-Operative Crohn's Disease

1. Identification of antibodies that may be associated with recurrence of Crohn's disease following intestinal resection
2. Correlation of these antibodies with phenotypic characteristics at baseline
3. Assessment of the potential use of serologic markers of mucosal healing and their correlation with endoscopic activity

4. Investigation of the relationship between faecal Calprotectin and serologic antibody responses in the post-operative setting

1.6.2.2 The Post-Operative Faecal Microbiome in Crohn's Disease

1. Characterisation of the faecal bacterial microbiota in post-operative Crohn's Disease at surgical resection and post-operatively, and investigation of the temporal changes in composition
2. Examination of differences in the faecal-associated microbiota between patients who have disease recurrence as compared to patients without disease recurrence
3. Investigation of possible microbial causes of Crohn's disease recurrence previously detected in the mucosally-associated microbiota such as *Proteus* spp. and low abundance of *F. prausnitzii*

1.6.2.3 Proteus species in Gastrointestinal Disease

1. To perform a systematic review of the literature to determine prior evidence linking *Proteus* spp. with gastrointestinal disease, with special reference to Crohn's Disease aetiology
2. To generate new hypotheses for the potential role of *Proteus* (and other members of the *Enterobacteriaceae*) in Inflammatory Bowel Disease at the mucosal interface
3. To review the possible effects of surgery on the relative abundance of *Proteus* spp. in post-operative Crohn's Disease patients.

1.7 Significance of this work

This will be the first prospectively-assessed surgical cohort of patients to undergo serial antibody testing with the aim of identifying factors that may predict endoscopic recurrence.

Chapter 4 represents the largest post-operative longitudinal assessment of the faecal microbiome in Crohn's Disease. It is the first large cohort to be comprehensively assessed using metagenomic techniques for fine resolution characterisation of the gut microbiome.

Chapter 5 is the first in-depth systematic investigation into the contribution of *Proteus* spp. to the aetiology of gastrointestinal diseases.

2 Study Design and Methods

2.1 The Clinical Post-Operative Crohn's Endoscopic Recurrence (POCER) Study

We have previously undertaken a randomised clinical trial to assess the value of structured pre-operative assessment to establish risk of disease recurrence, followed by a regime of selective prophylactic immunosuppression. The Post-Operative Crohn's Endoscopic Recurrence (POCER) study was undertaken in 17 hospitals around Australia and New Zealand, and recruited 174 patients who were then monitored for 18 months post-operatively.[6] Endoscopic evaluation was performed early to identify patients with evidence of disease, who then were given intensified drug therapy. Patients provided longitudinal blood, serum and stool samples for further study of the genetic, immunologic and microbial aetiology of Crohn's disease.

Analysis of clinical outcomes has already demonstrated a reduction in recurrence rates in patients who participated in the post-operative care algorithm, as well as the utility of monitoring disease using faecal biomarkers.[6, 7]

This study was conducted under the principles of good clinical practice and the supervision of the St Vincent's Hospital Human Research Ethics committee (HREC-A 077-09) and was registered with clinicaltrials.gov (NCT00989560). All patients provided written informed consent at enrolment. Ethical approvals for the main POCER study and the additional scientific analysis are shown in Appendix 3. The full study protocol is included in Appendix 2.

The POCER study was designed to answer a number of clinical questions regarding the aetiology, prevention and management of post-operative Crohn's disease recurrence. These included:

1. Evaluation of the structured longitudinal endoscopic monitoring of the anastomosis, with tailored drug intervention based on pre-operative risk factors for the following outcomes:
    a. Endoscopic disease progression
    b. Clinical disease recurrence (symptoms)
    c. Requirement for repeated surgery within the study period.

2. Quantify the possible benefits of therapeutic intensification with anti-TNF-α drugs in the post-operative period, especially in patients deemed as failures of standard immunosuppression (thiopurines).
3. To improve current post-operative scoring systems based on evidence from controlled trials (a “validated” scoring system).
4. To quantify any cost savings associated with reductions in recurrence rates.
5. Assessment of the clinical utility of faecal biomarkers in the early diagnosis of endoscopic recurrence.

Additionally, data and clinical samples were collected to address the following scientific questions detailed within this thesis:

1. Investigation of humoral immune responses (antibodies) pre- and post-operatively for predictive ability of endoscopic recurrence and temporal changes in the post-operative period.
2. The impact of, and changes within, the faecal microbiome post-surgery.
3. The influence of specific bacterial species previously identified in previously analysed mucosal samples from POCER patients.

2.2 Study Design

The prospective, randomised controlled POCER study was undertaken by 17 tertiary referral centres in Australia and New Zealand who routinely performed surgery on Crohn’s patients. Patients were recruited up to a month prior to surgery, and up to two weeks post-operatively. Overall, 174 patients were recruited between October 13, 2009, and September 28, 2011.[6] The study design is shown in Figure 2.1.

Patients were assigned at enrolment to a high or low risk stratification (Table 2.1) based on one or more risk factors (active smoking, previous intestinal resection and or perforating disease phenotype (Montreal B3)).

High Risk Patients
One or more of the following:
Patient is a current smoker
Patient has had a previous bowel resection for Crohn's Disease
Patient has perforating disease (abscess, perforation or fistula, previously or now) including perianal disease
Low Risk Patients
None of the above

Table 2.1 Risk Stratification Criteria for the POCER Study

Patients were enrolled prior to their baseline surgery on the assumption that they would continue to meet enrolment criteria following their operation. The inclusion and exclusion criteria are detailed in Table 2.2.

Inclusion Criteria
Patients with Crohn's disease who undergo resection with an endoscopically accessible primary anastomosis, which results in macroscopic normality.
Patients having a reversal of a temporary ileostomy created after previous surgery for Crohn's disease provided that the reversal of the ileostomy results in a primary anastomosis and macroscopic normality of the remaining bowel.
Patients with co-existing perianal disease provided the resection has led to a primary anastomosis and macroscopic normality of the intestine.
Patients must have proven history of Crohn's disease based on clinical, radiologic, endoscopic and histologic criteria.
Exclusion Criteria
Patients with anastomosis which is endoscopically inaccessible by standard colonoscopy.
Patients in whom there is persisting macroscopic abnormality post surgical resection.
Patients with Crohn's disease who have an end stoma (ileostomy or colostomy).
Patients for whom endoscopy is not suitable due to co-morbidities or unwell clinical state.
Inability to give informed consent.
Inability to obtain access to the anastomosis at colonoscopy.
Suspected perforation of the gastrointestinal tract.
Pregnancy.

Table 2.2 Inclusion and Exclusion Criteria for the POCER Study.

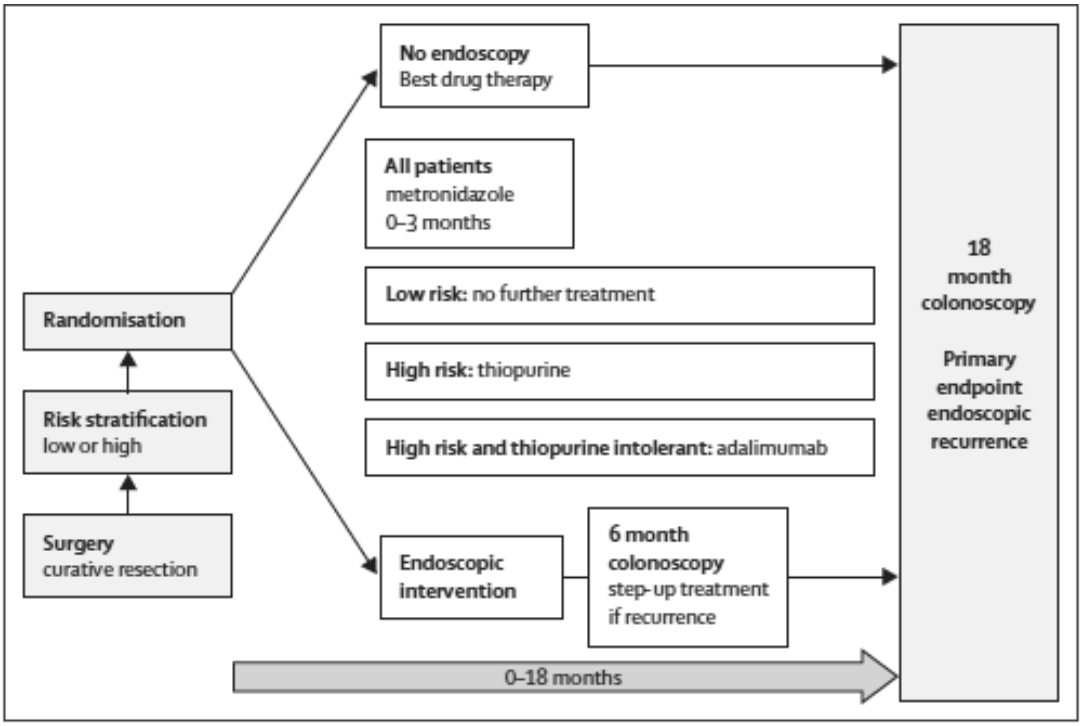


Figure 2.1 The POCER Study – Study Design[6]

All patients enrolled were prescribed metronidazole, 400mg BD. Dose reductions were permitted for intolerance, to 400mg mane, or if still not tolerated to 200mg BD for 3 months. Overall, 70% of patients tolerated the full prescribed dose of metronidazole.[6]

High risk patients additionally received a thiopurine (azathioprine 2mg/kg/day or 6-mercaptopurine 1.5mg/kg/day), or if intolerant to thiopurines, adalimumab at standard dosing (160mg at commencement, 80mg 2 weeks later, and 40mg each fortnight ongoing). Patients were instructed to taper any corticosteroids within 3 months of surgery.

Patients were then block randomised (by study site) in a 2:1 ratio to either active care (an endoscopy at 6 months) or standard best care (no endoscopy at 6 months). The primary endpoint was the Rutgeerts score (Table 2.3) at 18 months post-operatively.

2.2.1 Data Collection

Patients were monitored by phone at least monthly, and at each major visit (Baseline, Surgical Resection, 1 month, 6 months, 12 months, 18 months and unscheduled visits) and clinical parameters were assessed as per Table 2.4. Routine pathology was performed at each major visit as per the study protocol (Appendix 3).

Endoscopic assessment for the primary endpoint of the study was performed at each colonoscopy (with photographs), at 6 months (active care, 2/3 patients) and at 18 months in all patients.

Endoscopic recurrence is defined as a Rutgeerts score of ≥ i2 as shown in Table 2.3. Photographs of each grade are included in Appendix 1. The scoring was performed by two blinded investigators, on endoscopic photographs of the anastomosis (close and at a distance) as well as views of the ileum.

Rutgeerts Score	
i0	No macroscopic lesions
i1	≤ 5 apthous ulcers at the anastomosis
i2	> 5 apthous ulcers with normal mucosa between lesions or skip areas of larger lesions, or lesions confined to the anastomosis
i3	Diffuse apthous ileitis with inflamed mucosa
i4	Diffuse inflammation with larger ulcers or stenosis/narrowing of the anastomosis

Table 2.3 The Rutgeerts Score[3]

Data were recorded by study staff and recorded both in hard copy and in an electronic case record form (Episoft, Sydney, Australia). Following completion of the study by the final patient, this database was locked and extensively monitored for accuracy by Soula Krejany, Clinical Scientist.

Data were extracted from the database, cleaned in Excel and analysed in STATA except where otherwise stated.

Study Forms/ Assessments and Recordings	Baseline	Surgical Resection	1/12	2/12	4/12	6/12	7/12	8/12	10/12	12/12	15/12	18/12	24/12	Unscheduled Visit
Study Visits – In Person	X	X	X			X				X		X		X
Study Visits – By Phone or In Person				X	X		X	X	X		X		X	
Study Eligibility and Informed Consent	X													
Contact Information and Demographics	X													
Risk stratification & randomisation	X													
Pre-Operative Medications		X												
Patient History	X													
CDAI	X					X				X		X		X
Current Medications	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Health Care Resource Utilization	X		X	X	X	X	X	X	X	X	X	X	X	X
IBDQ and SF-36	X					X				X		X		

Study Forms/ Assessments and Recordings	Baseline	Surgical Resection	1/12	2/12	4/12	6/12	7/12	8/12	10/12	12/12	15/12	18/12	24/12	Unscheduled Visit
Symptom Assessment	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Routine Blood Investigations	X		X	X	X	X	X	X	X	X	X	X		X
Study Specific Blood Tests and Study Faecal Calprotectin	X					X				X		X		
Endoscopic Assessment						X active arm only						X		+/-
Adverse Events	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Anti-TNF Safety Screening	If indicated	If indicated				If indicated							X	
Drop-out and Treatment Failure	If indicated	If indicated	If indicated	If indicated	If indicated	If indicated	If indicated	If indicated	If indicated	If indicated	If indicated	If indicated		X

Table 2.4 The Visit and Investigations Schedule for the POCER Study

2.3 Clinical Sample Collection

2.3.1 Faecal Samples

Baseline faecal samples were obtained up to a month pre-operatively. Patients were counselled on collecting the specimen, storing it immediately (in a home freezer at -20°C). Patients then transported the sample on ice to the study staff, where they were immediately stored at -80°C. Patients also provided faecal samples using the same methodology, prior to endoscopic assessment, and were instructed to obtain this sample prior to the bowel preparation. Following collection of all samples, the frozen specimens were aliquoted using sterile technique (ensuring both the inner and outer layers were sampled) for the purposes of faecal Calprotectin assessment[7] and microbial sequencing,. DNA fixation solutions such as RNALater were not used. Samples were shipped on dry ice to the Murdoch Children's Research Institute for DNA extraction and sequencing. Further laboratory methods are detailed in section 2.10, and in chapter 4.

2.3.2 Blood Samples

Study blood samples (as opposed to routine blood testing) were obtained at each major study visit (baseline, 6, 12 and 18 months). 12ml of whole blood (3 x EDTA tubes) and 27ml of blood for serum separation (3 x Serum Separator tubes; SST) was collected at each time point. The whole blood was immediately frozen at -80°C. The SST tubes were spun at 2000rpm for 10min in a refrigerated centrifuge. The serum was then aseptically aspirated and stored at -20°C for serologic analysis. Prior to analysis, the serum was thawed and re-aliquoted for serologic analysis. These aliquots were then shipped on dry ice to Prometheus Laboratories, San Diego, California, USA.

2.4 Study Hypotheses

The overall hypotheses of the POCER Study (as detailed in the Study Protocol, page 11) were as follows, with the hypotheses related to this work **highlighted**:

> That a prospective endoscopically-guided management programme, with pre-determined therapeutic interventions, will lessen the severity of disease recurrence, health care resource use, and the need for further surgery in patients operated on for Crohn's disease.
>
> That specific changes in gut mucosal micro-flora at a Crohn's anastomosis cause disease recurrence.

That early changes in immune cell function / activity reflect sensitization to microbial flora, and that this activation differs in those with recurrence from those without recurrence.

That a reproducible, validated endoscopic scoring system will enhance post-operative management and will serve as a useful tool in studies of prevention of post-operative recurrence.

That anti-TNF therapy will lessen recurrent disease severity in patients resistant to, or intolerant of, standard immunosuppressive therapy.

That faecal calprotectin is a reliable bio-marker of disease recurrence.

2.5 Study Aims and Objectives

The overall aims and objectives of the POCER Study (as detailed in the Study Protocol, page 11) were as follows, with the hypotheses related to this work **highlighted**:

The primary aim is to evaluate the effect of longitudinal endoscopic monitoring of the bowel anastomosis, with therapeutic intervention tailored to the severity of endoscopic disease recurrence, on: (a) endoscopic disease progression, (b) recurrent clinical symptoms, and (c) need for further surgery.

To prospectively characterise endoscopic, histologic, **microbiological and immunologic factors that are associated with disease recurrence at the anastomosis in patients having resectional surgery for Crohn's disease.**

To examine the benefit of anti-TNF therapy in modifying disease recurrence in patients with high risk of recurrence or patients who have failed standard immunosuppressive therapy.

To establish a reproducible scoring system for evaluating post-operative disease recurrence.

To evaluate the cost-benefit of endoscopic disease monitoring in post-operative Crohn's disease patients.

To assess faecal calprotectin as a non-invasive measure of disease recurrence.

2.6 Study Power Calculations

The statistical analysis of the clinical POCER study[6] was based on the following power calculations:

Alpha value = 0.05 (1-sided)

Power = 80%

Expected rate of the primary outcome (Rutgeerts Score $\geq$ i2) at 18 months for control group = 60%

Expected rate of the primary outcome at 18 months for active group = 35%

These calculations are based on a randomised, placebo-controlled trial of antibiotics (Metronidazole) and Thiopurines (Azathioprine) to prevent post-operative recurrence at one year post operatively.[49] A recurrence rate of 69% occurred in the antibiotic only arm, compared to 44% of patients on both drugs (Per Protocol Analysis; $P = 0.048$).[49]

The adalimumab arm was based on a placebo controlled randomised study of an anti-TNF-α drug (Infliximab) for the prevention of endoscopic recurrence, with severe recurrence occurring in 54% of the placebo arm versus 9% of patients on infliximab.[201]

Initially, this resulted in recruitment on a 2:1 ratio of 84 "active care" participants (endoscopic monitoring group) and 42 "standard care" controls, with an initial drop-out rate of 16%. Total recruitment was expected to be 100 active care and 50 standard care patients.

The power calculations were reviewed based on recruitment and retention (proportion of enrolled patients reaching primary endpoint) when the first 100 patients had met the six-month time point. Following this analysis, recruitment targets were based on a 31% treatment failure/withdrawal rate.

With revision, to allow for a 31% drop-out of subjects, we aimed to recruit 113 and 57 patients in both groups respectively, for a total of 170 patients.

2.6.1 Amendments to Scientific Analyses from Initial Protocol

With the initial POCER study protocol being approved in 2009, extensive amendments were required to the initial scientific protocol. These included moving from micro-array

and pyrosequencing for microbial analysis to the more detailed 16S-based techniques. Changes were also made to the serologic testing. Amendments to the Human research Ethics Committee approval were approved by Chairman's action. The final study protocol (following an initial amendment) is included in Appendix 2. The ethics expansion request and approvals covering all aspects of this thesis are included in Appendix 3.

2.7 Intervention and Primary Endpoint

2.7.1 Endoscopic Intervention

The primary endpoint of the POCER study was the presence and severity of endoscopic recurrence 18 months after surgery using the Rutgeerts score.

The primary outcome was reported in De Cruz *et al* (2015).

"At 18 months, endoscopic recurrence occurred in 60 (49%) patients in the active care group and 35 (67%) patients in the standard care group (p=0·03). Complete mucosal normality was maintained in 27 (22%) of 122 patients in the active care group versus four (8%) in the standard care group (p=0·03). In the active care arm, of those with 6 months recurrence who stepped up treatment, 18 (38%) of 47 patients were in remission 12 months later; conversely, of those in remission at 6 months who did not change therapy recurrence occurred in 31 (41%) of 75 patients 12 months later."[6]

2.8 Datasets Obtained

The data obtained from participants in the POCER Study are shown in Figure 2.2. Not all patients provided all clinical samples at each time point.

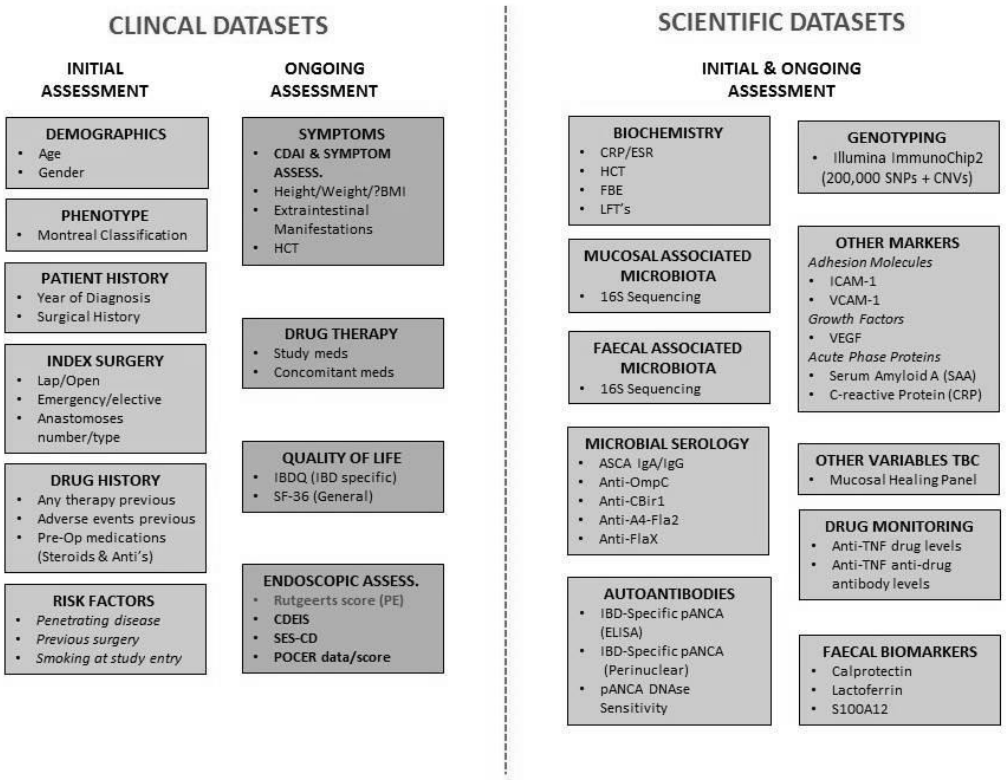


Figure 2.2 Clinical and scientific datasets obtained within the POCER Study.

2.9 Serology

Serologic testing for reactivity to self- and microbial- antigens was undertaken in partnership with Prometheus Laboratories, San Diego, USA, using their proprietary serologic panel, the IBD-SGI test.

2.9.1 IBD-SGI Panel

The IBD-SGI panel measures the antibodies detailed in Table 2.5.

Marker	Type	Antigen	Ref. Range (EU/ml)	Ref.
ASCA-IgA	Anti-*Saccharomyces cerevisiae* IgA	Mannose peptides from phosphopeptidomannan derived from bakers/brewers yeast	5.0	[75]
ASCA-IgG	Anti-*Saccharomyces cerevisiae* IgG		13.3	[75]
ANCA-IgG	Anti-neutrophil Cytoplasmic Antibody IgG	Self-reactivity to a constituent of neutrophil granules	20.0	[79]
pANCA	Perinuclear anti-neutrophil cytoplasmic antibody staining	A subgroup of ANCA, based on perinuclear immunofluorescence staining in addition to ELISA	Not Detected	[79]
DNAse-sensitive pANCA	Deoxyribonuclease - sensitive Perinuclear anti-neutrophil cytoplasmic antibody staining	As above, but response is lost after DNAse digestion of fixed neutrophils	Not Detected	
Anti-CBir1	Anti-flagellin antibody	*Clostridium* subphylum XIVa cluster of Gram-positive bacteria	38.6	[67, 71]
Anti-OmpC	Anti-outer membrane protein antibody	Outer membrane porin protein from *Escherichia coli*	11.5	
Anti-A4-Fla2	Anti-flagellin antibody	*Clostridium* subphylum XIVa cluster of Gram-positive bacteria	40.9	[77]
Anti-Fla-X	Anti-flagellin antibody	Outer membrane porin protein from *Escherichia coli*	30.5	[67]

Table 2.5 Antibodies measured in the IBD-SGI Panel

Testing was carried out by Prometheus scientists, blinded to patient identity, randomisation and treatment, using a standardised ELISA performed on a Freedom EVO 200 liquid-handling robot (Tecan, Männendorf, Germany). Reference ranges were as reported in Choung et al (2016), and were calculated based on 722 serum samples from 200 healthy controls and 522 patients with non-IBD gastrointestinal disease. Intervals for each microbial antibody were defined according to the guidelines of Clinical and Laboratory Standards Institute.

Titres (ELISA units per ml; EU/ml) were measured relative to a "5-parameter, logistic-derived 6-point standard curve derived from standards prepared from a pool of sera".[202]

Assessment for atypical perinuclear-ANCA staining (pANCA) was done on ANCA titre-positive samples, and performed by immunofluorescence on DNAse treated and untreated alcohol fixed neutrophil slides, as previously described.[202, 203]

Data was returned to the POCER study team and statistical analysis was performed as per Chapter 3.

2.10 Faecal Microbiome

2.10.1 DNA extraction and PCR

Following receipt of the frozen faecal samples, samples were batched and underwent DNA extraction with additional bead-beating steps using the Mo-Bio PowerSoil DNA extraction kit (Mo Bio Laboratories, Carlsbad, USA)

Briefly, 100-200 mg of each stool sample was added to sterilised tubes with 500µl of Bead Solution (PowerBeads), mixed and added to a PowerBead Tube. Tubes were briefly vortexed, and 60 µl of Solution C1 added and samples were then incubated at 65°C for 10 minutes in a waterbath. Samples were then vortexed at 1000m/s for 10 minutes and further incubated at room temperature for 2 hours, with vortexing at 1000m/s for 10 minutes once every 20 minutes. Samples were then centrifuged at 13,000 x g for 1 minute and the supernatant transferred to a clean tube. 250 µl of Solution C2 was then added to the supernatant and vortexed for 5 seconds, followed by incubation on ice for 5 minutes, and centrifugation at room temperature for 1 minute at 13,000 x g. Supernatant was again transferred to a clean tube and 200 µl of Solution C3 was added, tubes were vortexed briefly and incubated on ice for 5 minutes. The samples were then centrifuged again at room temperature for 1 minute at 13,000 x g and again, the supernatant was transferred to a clean tube. 1200µl of Solution C4 was

then added to the supernatant and vortexed for 5 seconds. The solution was then loaded on to the spin column (in 650µl aliquots) and centrifuged at 13,000 x g for 1 minute at room temperature. Flow-through was discarded and the next aliquot added and the centrifugation step repeated. 500 µl of Solution C5 was then added to the column for the filter wash step and centrifuged at room temperature for 1 minute at 13,000 x g, the flow-through discarded and centrifugation repeated. After placing the filter in a clean collection tube, the DNA was eluted with 100 µl of sterile DNA-Free PCR Grade Water, and spun for 1 minute at 13,000 x g. DNA concentration was tested using the Nanodrop Spectrophotometer (Thermo Scientific, Waltham, USA). The eluted DNA was then stored frozen (-20° to -80°C).

The V2 region of the small ribosomal subunit (16S) was then amplified using primers F101 and 342R using polymerase chain reaction [PCR] with Illumina index/adaptors using the Expand High Fidelity PCR kit (Roche Diagnostics, Mannheim, Germany) (Table 2.6). *Escherichia coli* genomic DNA was used as positive control, and PCR grade water as the negative control.

PCR cycle conditions

Stage I:	94°C	3min
Stage II:	94°C	30sec
	58°C	30sec
	72°C	45sec
	Total	35 cycles
Stage II:	72°C	7min
	4°C	forever

The DNA was then purified on a 1% TBE Agarose gel to remove unincorporated primers. Bands were excised using the positive control and DNA size ladder to identify band of interest.

Illumina Adapter	8-nt index sequence	10-nt pad sequence	2-nt linker	Bacterial variable region V2 forward primer
AATGATACGGCGACCACCGAGATCTACAC	AAGCAGCA	TATGGTAATT	GT	AGYGGCGACGGGTGAGTAA
Reverse Primer				
Illumina Adapter	**8-nt index sequence**	**10-nt pad sequence**	**2-nt linker**	**Bacterial variable region V2 reverse primer**
CAAGCAGAAGACGGCATACGAGAT	ACCTAGTA	AGTCAGTCAG	CC	CYACTGCTGCCTCCCGTAG

Table 2.6 Sequencing Primers for Amplification of the V2 Region of the 16S rRNA gene.

The excised bands were then purified using the Wizard SV Gel and PCR Clean-Up System (Promega Corporation, Madison, USA) according to protocol.

96 well plates were prepared in duplicate for Illumina MiSeq sequencing by Australian Genome Research Facility (AGRF). All runs were performed as 250bp paired end reads (251 cycles, 8 cycles and 251 cycles).

2.10.2 Illumina Output Quality Control Pipeline

Sequencing of the 314 initially obtained samples required four 96-well plates to be sequenced. Initial assessment of plates 3 and 4 (representing 122/314 samples) demonstrated that the sequencing quality was reduced when compared to the other plates (1 and 2), as a result the duplicate plate for these samples was sequenced. All raw data from all six plates (total) underwent quality assurance (436 samples sequenced of which 122 were performed in duplicate).

The FASTQ files were assessed for sequencing quality was assessed using FastQC[204], on the basis of Phred score, n base calls and number of high quality reads obtained. The Phred Quality score (Q score) is a measure of base call accuracy, with a Phred score of 30 indication a 1/1000 chance that the base call at a particular position is incorrect.[205] A score of 30 is generally agreed to be the minimum acceptable threshold for per-base accuracy.[206] The data was parsed into Python (Version 2.7.10; https://www.python.org) to enable more detailed assessment of read quality, including uncalled bases per position, Phred scores and to determine the number of sequences remaining post QC.

Figure 2.3 illustrates the lower read numbers obtained from the original plate 3 and 4 sequencing, and the relative difference between the original plates and the re-sequenced plates.

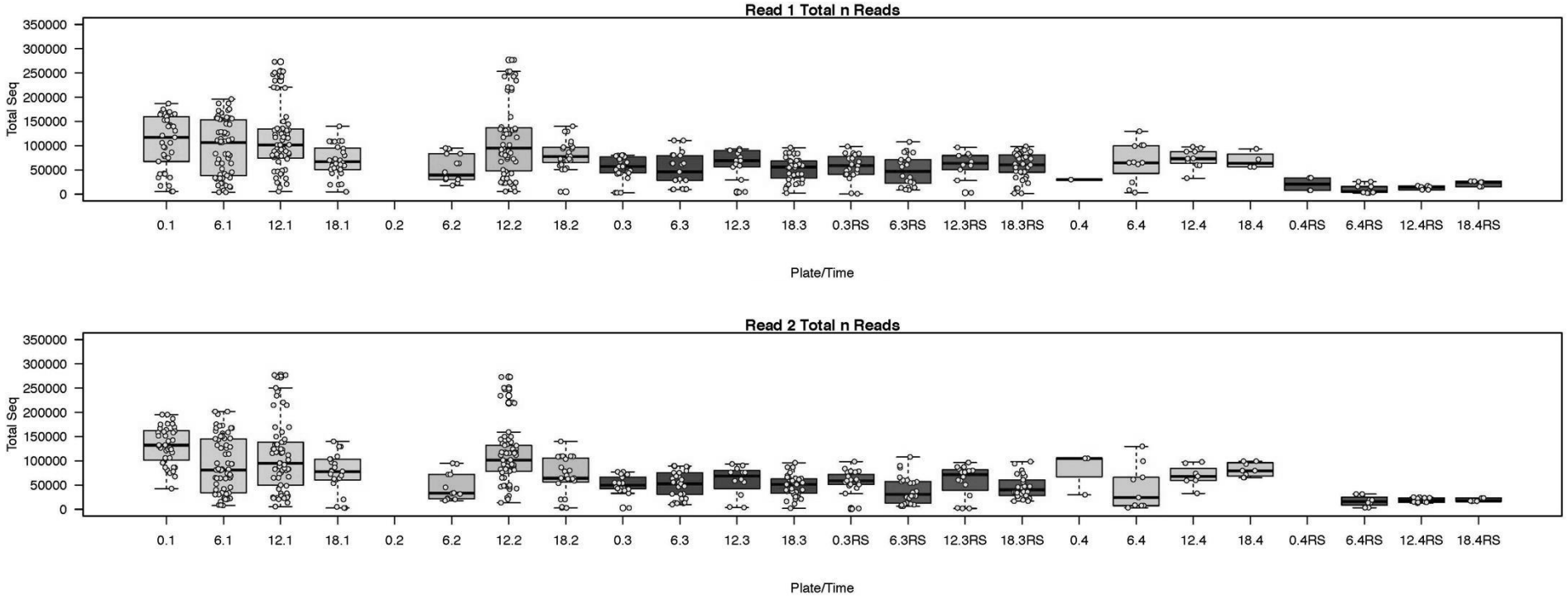


Figure 2.3 Boxplot showing the total number of reads (Read 1 and Read 2) across all sequenced plates following FASTQC.[204] X-axis is time point and plate (Time 0,6,12,18; Plates 1,2,3,4,3RS,4RS). Y-axis is the number of reads. RS – Resequenced

Following this, the number of null (N) base calls (where the sequencer is unable to determine the identity of the base at position x was also assessed. The read 2 n basecalls was higher for the original sequencing of plate 4, and justified re-sequencing of this plate. Plate 3 was re-sequenced due to lower total read numbers.

All data (both initial and re-sequenced samples) were assessed for quality by a comprehensive processing pipeline. All reads in all samples were trimmed to 200bp by FASTX-Toolkit Version 0.0.14 (http://hannonlab.cshl.edu/fastx_toolkit/). Merging of the paired reads was undertaken with FLASH, with a minimum overlap of 100bp and max of 200bp, and a read length of 200bp.[207] Pre- and post-merge read counts were parsed and compared, with a threshold set of <15,000 pre-merge reads, with a >90% merge percentage required. On the basis of the post-merge quality control, it was decided to use the original merged plate 3 data (not the re-sequenced plate) and for plate 4 data, the read counts for the original plate were acceptable but the merge percentages were poor. As such, the decision was made to clip the primer (using FASTX-Toolkit as per above) from the R1 read and to use these 23 samples without merging. The sequencing plate was recorded for each sample, and used as an adjusting factor in all downstream analysis. 291 samples (Table 2.7) progressed to demultiplexing in QIIME (using the *split_libraries_fastq.py* script, allowed n's = 1).[208]

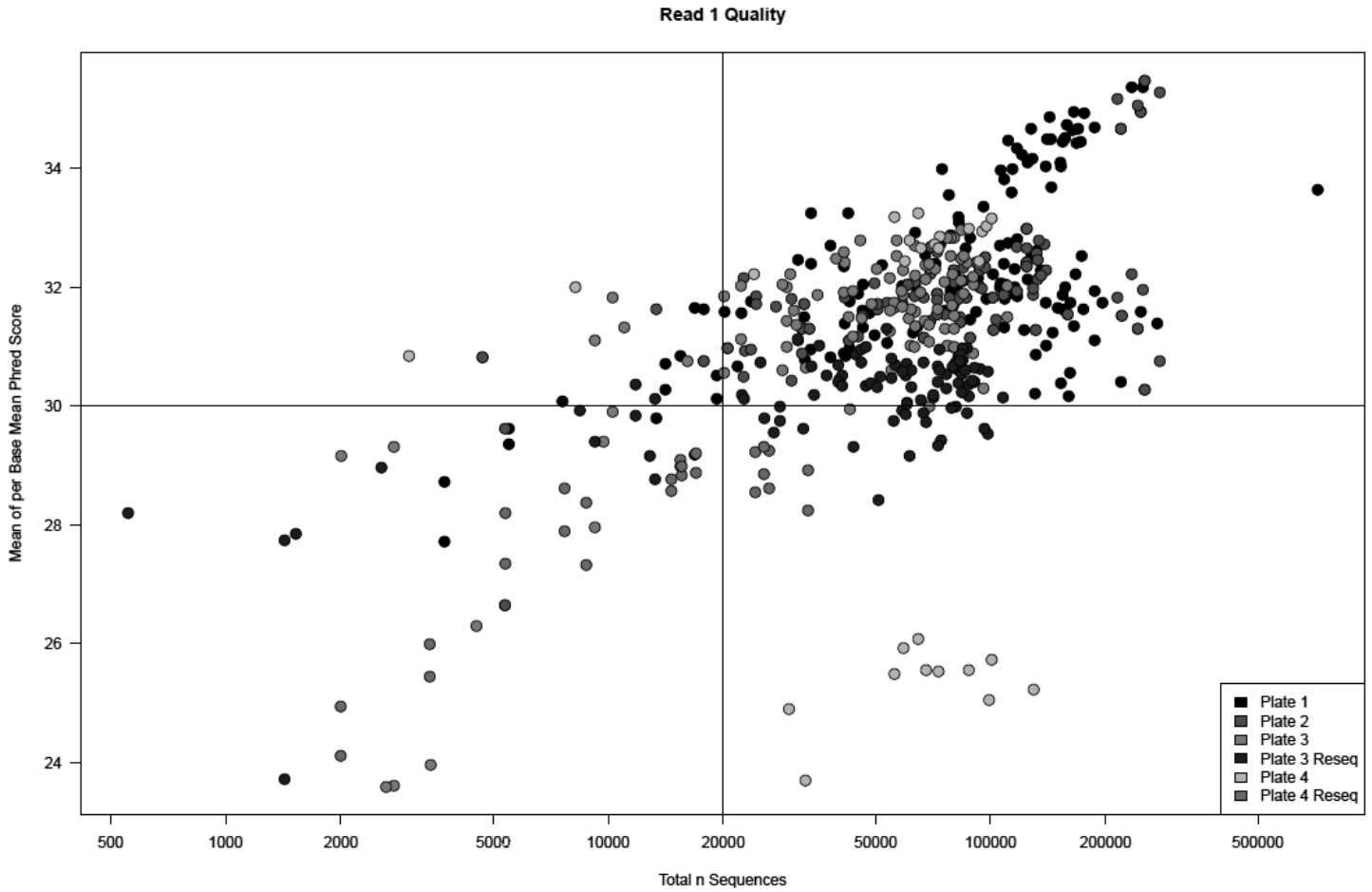


Figure 2.4 Plot of mean (of per base mean) Phred score for read 1 vs. total n sequences for all plates sequenced.

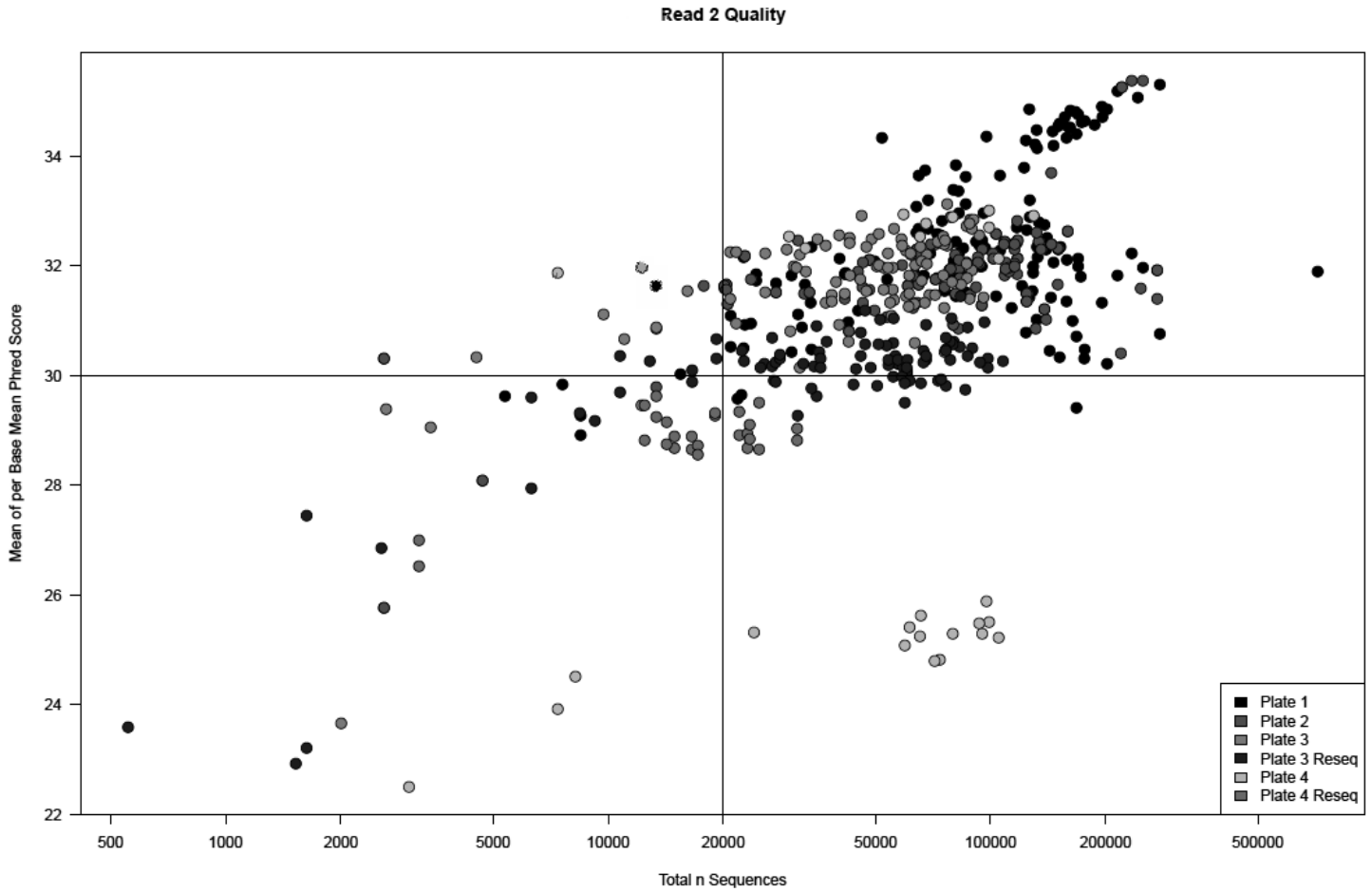


Figure 2.5 Plot of mean (of per base mean) Phred score for read 2 vs. total n sequences for all plates sequenced.

Following the merge, a closed reference pick was performed as a first-pass analysis (pick_closed_reference_otus.py, using Greengenes Version 13_05, 97%) to confirm quality control data and allow for final sample selection. Three samples did not meet quality threshold following the close reference pick, with match percentages less than 35% of pre-reference pick reads (Table 2.7).

Meanwhile, the output of the *split_libraries_fastq.py* script was passed to the *identify_chimeric_seqs.py* script to identify chimeras using a reference-based approach. The UCHIME v4.2 method (http://drive5.com/usearch/usearch_docs.html) was used referencing against the rdp-Gold database.[209] These sequences were then removed using the *filter_fasta.py* script.

Of the 288 samples that met quality thresholds and progressed to chimera removal, 1,369,078 (6.54%) reads were removed, leaving a total of 23,577,525 reads for analysis.

These reads were then assigned to OTUs using the subsampled open reference method within the *pick_open_reference_otus.py* script in QIIME.[208] This script uses the Greengenes 97% OTU reference set, version 13_8[210], to remove reads with less than 60% sequence identity, with 22491263 reads remaining (average reads 78095 per sample, range 10701-665671).

	n samples sequenced	Fail Initial QC	Progressed to Demultiplexing	Failed Close Ref QC	Final n Samples for Open Ref Picking
Plate 1	96	5	91	1	90
Plate 2	96	4	92	2	90
Plate 3	96	11	85	-	85
Plate 4 (R1 ONLY)	26	3	23	-	23
Plate 3RS	*96*	*Original Used (96)*	-	-	-
Plate 4RS	*26*	*Original Used (26)*	-	-	-
Total	**436**	**23**	**291**		**288**

Table 2.7 Sample progression through the OTU quality control and picking pipeline.

Following the subsampled open reference picking (including de novo picking), generation of the OTU table and taxonomic assignment, analysis progressed as per the steps outlined in chapter 4.

3 Serologic antibodies in relation to outcome in postoperative Crohn's disease

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Serologic antibodies in relation to outcome in postoperative Crohn's disease

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Key words
antibodies, Crohn's disease, postoperative, serology, smoking.

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Author contributions: A. L. H., P. D. C., and M. A. K. – study concept and design; acquisition of data; analysis; data interpretation; drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical analysis; and obtained funding. F. S. and F. P. – sample analysis and critical revision of the manuscript for important intellectual content. A. G. and D. L. – statistical analysis, interpretation of data, and drafting of the manuscript. J. M. A., P. A. B., M. P. S., T. H. F., P. R. G., H. D., R. B. G., R. W. L., F. A. M., I. K., G. R. S., W. S., S. J. B., S. J. B., and W. R. C – acquisition of data and critical review of manuscript for important intellectual content.

Abstract

Background and Aim: Disease recurs frequently after Crohn's disease resection. The role of serological antimicrobial antibodies in predicting recurrence or as a marker of recurrence has not been well defined.
Methods: A total of 169 patients (523 samples) were prospectively studied, with testing peri-operatively, and 6, 12 and 18 months postoperatively. Colonoscopy was performed at 18 months postoperatively. Serologic antibody presence (perinuclear anti-neutrophil cytoplasmic antibody [pANCA], anti-*Saccharomyces cerevisiae* antibodies [ASCA] IgA/IgG, anti-OmpC, anti-CBir1, anti-A4-Fla2, anti-Fla-X) and titer were tested. Quartile sum score (range 6–24), logistic regression analysis, and correlation with phenotype, smoking status, and endoscopic outcome were assessed.
Results: Patients with ≥ 2 previous resections were more likely to be anti-OmpC positive (94% *vs* 55%, ≥ 2 *vs* < 2, $P = 0.001$). Recurrence at 18 months was associated with anti-Fla-X positivity at baseline (49% *vs* 29%; positive *vs* negative, $P = 0.033$) and 12 months (52% *vs* 31%, $P = 0.04$). Patients positive ($n = 28$) for all four antibacterial antibodies (anti-CBir1, anti-OmpC, anti-A4-Fla2, and anti-Fla-X) at baseline were more likely to experience recurrence at 18 months than patients negative ($n = 32$) for all four antibodies (82% *vs* 18%, $P = 0.034$; odds ratio 6.4, 95% confidence interval 1.16–34.9). The baseline quartile sum score for all six antimicrobial antibodies was higher in patients with severe recurrence (Rutgeert's i3-i4) at 18 months, adjusted for clinical risk factors (odds ratio 1.16, 95% confidence interval 1.01–1.34, $P = 0.039$). Smoking affected antibody status.
Conclusions: Anti-Fla-X and presence of all anti-bacterial antibodies identifies patients at higher risk of early postoperative Crohn's disease recurrence. Serologic screening pre-operatively may help identify patients at increased risk of recurrence.

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Introduction

Within a year of Crohn's disease resection, recurrence occurs in up to 90% of patients.[1,2] Further surgery is required in a majority of patients within 10 years.[3] We have recently demonstrated in a prospective study that smoking, penetrating disease, and previous surgery are associated with an increased risk of earlier disease recurrence.[4] Biomarkers to identify patients at risk of recurrence would be valuable in focusing preventive therapy.

Circulating antibodies in inflammatory bowel disease (IBD) are directed against auto-antigens or enteric microbial antigens. Serological antibodies have been studied in relation to need for surgery[5–10] and development of more complex disease.[9–15] Choung *et al.* have recently shown that complex Crohn's disease patients often have serologic antibodies present pre-diagnosis and higher titers when compared with patients with uncomplicated disease.[16] However, there are limited data on whether such antibodies may predict the development of, or are associated with, recurrent disease after surgery.[17,18] Previous studies have demonstrated that anti-*Saccharomyces cerevisiae* antibodies (ASCA) alone are not sufficient to predict recurrence.[18–20] A small prospective study found that ASCA IgG and IgA did not predict the need for further surgery.[17] The development of novel serological IBD-related antibodies provides an opportunity to investigate this further.[21,22]

Microbial antigens known to elicit an antibody response in IBD include oligomannan, cell wall porin proteins, and flagellin subunits. Antibodies to mannan cell wall proteins derived from baker's yeast, *Saccharomyces cerevisiae* (ASCA IgG or IgA), are highly prevalent in Crohn's disease.[23,24] Omp-C is a bacterial outer membrane protein derived from *Escherichia coli*.[14] The antigens CBir1, A4-Fla2, and Fla-X are flagellin subunit proteins linked to *Clostridium* cluster XIVa.[25,26]

In a randomized controlled trial examining the optimal strategy for preventing recurrence of Crohn's disease after intestinal resection (postoperative Crohn's endoscopic recurrence [POCER] study),[4] patients were stratified according to clinical risk factors, treated to prevent recurrence, and monitored for recurrence using ileo-colonoscopy and faecal calprotectin measurement.[4,27] We now aim to assess the relationship between the presence and magnitude of serologic antibodies and the postoperative course in this cohort of patients.

Materials and methods

The POCER Study was a prospective, randomized, multi-center trial in 174 patients undergoing resection of all macroscopic luminal Crohn's disease.[4] Patients were stratified as high risk if they had one or more of previous resection, smoking, or perforating disease, or low risk for those with no risk factors. Patients were randomized (2:1 ratio) to a colonoscopy at 6 months (active care) or to best standard drug therapy. All patients underwent a colonoscopy at 18 months.

Patient phenotype was classified at baseline according to the Montreal classification,[28] disease activity assessed using the Crohn's disease activity index (CDAI), and all medications recorded. Smoking history was assessed as current, past, or never smoker at baseline. All patients received metronidazole (400 mg BD) for 3 months; high-risk patients additionally received a thiopurine (azathioprine 2 mg/kg or 6-mercaptopurine 1.5 mg/kg daily) or adalimumab (160 mg initially, 80 mg at 2 weeks, and then 40 mg fortnightly thereafter) if thiopurine intolerant.

Endoscopic assessment was undertaken using the Rutgeerts Score,[29] with recurrence defined as a score $\geq$ i2. Patients in the active care arm with endoscopic recurrence at 6 months received intensified drug therapy: low-risk patients stepped up to a thiopurine, and patients on a thiopurine commenced combination therapy with the addition of adalimumab 40 mg fortnightly. Patients on adalimumab therapy intensified dosing to 40 mg weekly.

Five hundred and twenty-three serum samples for antibody testing were taken from 160 patients at baseline (peri-operatively, prior to or within 4 weeks of surgery), 142 patients at 6 months, 111 patients at 12 months, and 110 patients at 18 months postoperatively.

Table 1 Patient demographics at baseline

Serology cohort (at least one measurement) *n* = 169	All	
	n	%
n (Male)	77	46
Age > 40 years	74	44
Age, median (years):		
Inter quartile range (IQR)	36 (26–46)	
Age at diagnosis (years):		
≤ 16 Years	19	11
17–40 Years	129	76
> 40 Years	21	12
Duration of Crohn's disease (years):		
median (IQR)	9 (4–16)	
≥ 10 years	66	39
Disease location at surgery:		
Ileum only (L1)	92	54
Colon only (L2)	11	7
Ileum and colon (L3)	66	39
Disease phenotype at surgery:		
B1 (Inflammatory)	16	9
B2 (Stricture)	60	36
B3 (Penetrating)	93	55
Indication for surgery:		
Failure of drug therapy	37	22
Obstruction	48	28
Perforation	84	50
Number of prior surgical resections:		
0	120	71
1	32	19
2	9	6
3 or more	8	5
Smoking status:		
Active smoker	52	31
Past smoker	41	24
Never smoker	76	45
Immediate postoperative baseline drug therapy:		
Metronidazole alone	27	16
Thiopurine	99	59
Adalimumab	43	25
Baseline CDAI *n = 148*		
CDAI > 150	110	74
CDAI > 200	88	59

CDAI, Crohn's disease activity index.

Overall, 169 of the 174 patients provided one or more serum samples. Baseline characteristics of the cohort are shown in Table 1.

Antibody testing. Antibody testing was performed using a commercial enzyme-linked immunosorbent assay (ELISA; IBD-SGI panel, Prometheus Laboratories, San Diego, CA). The panel measures the following antibodies: anti-neutrophil cytoplasmic antibody (ANCA) titer and perinuclear staining pattern (pANCA), anti-*Saccharomyces cerevisiae* antibodies (ASCA IgA and IgG), anti-CBir1 IgG, anti-OmpC IgA, anti-A4-Fla2 IgG, and anti-Fla-X IgG. Results were expressed as ELISA units (EU/ml), with positivity assessed according to the reference ranges defined in Choung *et al.*[16] Testing was performed blinded to patient data. Assessment for atypical perinuclear-ANCA staining (pANCA) was performed on ANCA titer positive samples, by immunofluorescence on DNAse treated and untreated alcohol fixed neutrophil slides, as previously described.[13,30]

Statistical analysis. Data were analysed using STATA Version 12 (StataCorp, Texas, USA). Associations between antibody positivity and patient characteristics were assessed using Chi^2 or Fisher's exact test, while the association between antibody levels and patient characteristics were assessed using the Kruskal–Wallis test. Correlations between continuous variables were assessed using Spearman's rank correlation. Associations between binary outcomes for endoscopic recurrence, serologic markers, and phenotype were determined using logistic regression.

The cumulative magnitude of antibody responses for all six tested antimicrobial antibodies (ASCA IgA and IgG, anti-CBir1 IgG, anti-OmpC IgA, anti-A4-Fla2 IgG, and anti-Fla-X IgG) was investigated using the quartile sum score method (range 6–24) as previously described.[31]

Anti-neutrophil cytoplasmic antibody titer and pANCA fluorescence (positive or negative) were excluded from the quartile sum score analysis, but pANCA was used as an adjusting factor in subsequent analyses.

The mean quartile sum score and the sum of the number of positive markers were compared between groups using the two-sample *t*-test and included in a logistic regression model. Comparisons were corrected for gender, age, disease location, smoking, and pANCA status, in relation to endoscopic recurrence.

Positive predictive values and negative predictive values were determined for single antibodies, combinations, quartile sum score, and number of positive markers, for prediction of endoscopic recurrence. Receiver operator characteristic curves were plotted (sensitivity *vs* 1-specificity), and the area under the curve were calculated (AUROC).

Ethical considerations. The POCER study, including the collection and analysis of serological samples, was approved by the Human Research Ethics Committee of St Vincent's Hospital, Melbourne (HREC-A 077/09), and is registered with ClinicalTrials.gov (NCT00989560). All patients provided written informed consent.

Results

Presence of serological antibodies. Of the 160 patients with baseline testing, 24 of 160 (15%) were positive for pANCA, 158 of 160 (99%) for ASCA IgA, 75 of 160 (47%) for ASCA IgG, and 74 of 160 (46%) for both ASCA IgA and IgG.

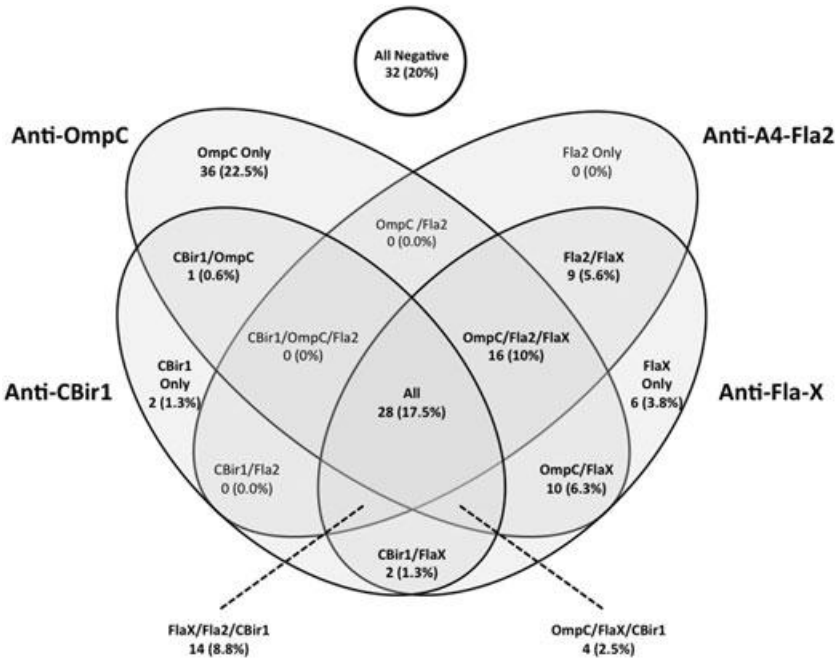

Figure 1 Overlapping bacterial antibody positivity at baseline.

With regard to anti-bacterial antibodies, 95 of 160 (59%) of patients were positive for anti-OmpC, 51 of 160 (32%) for anti-CBir1, 67 of 160 (42%) for anti-A4-Fla2, and 89 of 160 (56%) for anti-Fla-X.

Thirty-two of 160 (20%) of patients were negative for all bacterial antibodies, and 28 of 160 (17.5%) were positive for all four. Overlapping antibody positivity to the bacterial antigens is shown in Figure 1. The largest group of patients (22.5%) was positive only for anti-OmpC.

Ninety-three percent of patients had ileal or ileo-colonic disease (Table 1). Disease phenotype according to the Montreal classification[28] was associated as follows: pANCA positively associated with inflammatory (B1) disease (odds ratio [OR] 6.1 [95% confidence interval {CI} 1.8–20.3], $P = 0.003$) and negatively associated with penetrating (B3) disease (OR 0.4 [95% CI 0.14–0.90], $P = 0.029$). Anti-OmpC was negatively associated with inflammatory (B1) disease (OR 0.27 [95% CI 0.08–0.93], $P = 0.038$).

Change in antibodies over time. There was no significant change in mean quartile sum score (all antibodies combined for each patient) between baseline and 18 months ($\Delta -0.32$, $P = 0.265$). However, the mean number of positive markers changed significantly over time ($\Delta -0.33$, $P = 0.006$). The mean titer for each antibody changed significantly from baseline to 18 months for all antibodies (Fig. 2), although the magnitude of change was small, between 3.24 EU/ml for ASCA IgG and −10.81 EU/ml for anti-Fla-X.

The median values for the change in each individual time period (0–6 months, 6–12 months, and 12–18 months) were assessed for variation in titer, to exclude the surgical resection as a cause of titer variation over time. There was significant variation in ANCA ($P = 0.015$), anti-OmpC ($P = <0.001$), and anti-Fla-X ($P = <0.001$) titers between each time-period. No marker was consistently stable across all time periods, although the magnitude of the change for all antibodies was small.

Relationship between serological antibodies, surgery, risk factors for recurrence, and presence of disease recurrence

Surgery. One hundred and eleven of the 160 (69%) patients with baseline measurements had no previous surgery. The 17 patients with two or more previous resections were significantly more likely to be anti-OmpC positive at baseline than the 143 patients with less than two previous resections (94% *vs* 55%, $P = 0.001$). Anti-A4-Fla2 was negatively associated with previous surgery (OR 0.43 [95% CI 0.2–0.9], $P = 0.025$).

With regard to the indication for current surgery, patients positive for anti-OmpC at baseline were more likely to undergo surgery for failure of medical therapy than those having surgery for another indication (OR 2.20, 95%CI 1.0–4.7; $P = 0.044$). Patients positive for anti-A4-Fla2 were more likely to have surgery for obstruction (OR 2.1, 95%CI 1.0–4.3; $P = 0.05$).

Prediction of postoperative endoscopic recurrence. Antineutrophil cytoplasmic antibody titer and atypical perinuclear staining (pANCA) at baseline were not associated with endoscopic recurrence at 18 months, with no difference between patients positive at baseline for ANCA titer or pANCA compared with patients who were negative: ANCA titer 45% *vs* 39%, $P = 0.600$, pANCA staining 35% *vs* 41%, $P = 0.632$.

Endoscopic recurrence at 18 months did not differ between patients positive at baseline for ASCA IgA and IgG compared with patients who were negative: ASCA IgA 40% *vs* 50%, $P = 1.00$, ASCA IgG 44% *vs* 37%, $P = 0.464$.

Patients with recurrence at 18 months were more likely than those without recurrence to be positive for anti-Fla-X when measured at all time points, with this being significantly more likely at baseline (49% *vs* 29%, $P = 0.05$) and 12 months (52% *vs* 31%, $P = 0.04$), but not significantly so at 6 (48% *vs* 33%, $P = 0.121$) or 18 months (51% *vs* 34%, $P = 0.115$).

Patients positive for all four antibacterial antibodies (anti-CBir1, anti-OmpC, anti-A4-Fla2, and anti-Fla-X) at baseline were more

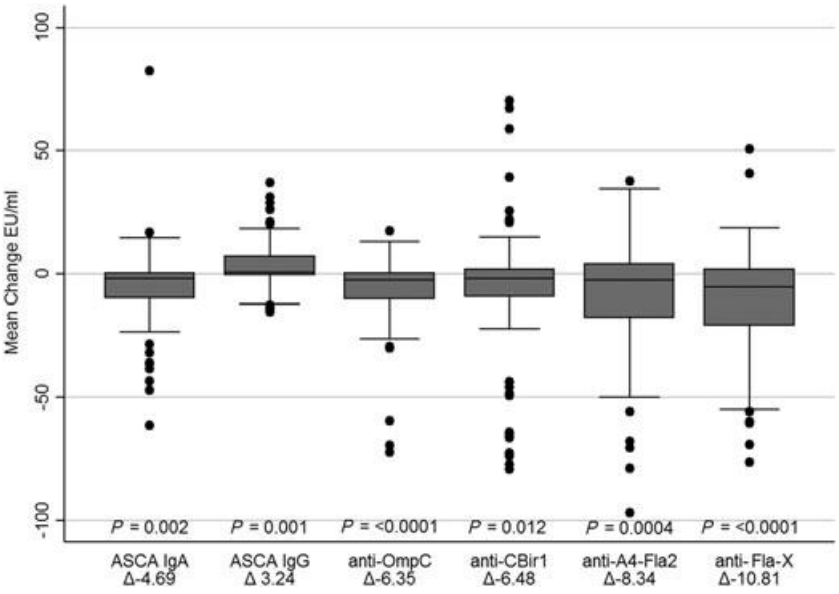

Figure 2 Change in mean antibody titers from baseline to 18 months (EU/ml) for each antibody tested. *P* values denote statistically significant variation in mean values (one sample *t*-test), with the change (delta, Δ) in EU/ml shown beneath the x-axis. All boxes denote median and inter quartile range.

Table 2 Mean quartile sum score and mean number of positive markers measured at baseline, 6, and 18 months for the prediction of endoscopic outcome (recurrence or remission) at 18 months

	Mean values		
Baseline serology for 18-month endoscopic outcomes	Remission		Recurrence
Mean quartile sum score	14.86		16.31
P value		0.067	
Mean *n* positive markers	3.18		3.82
P value		0.038	
6-month serology for 18-month endoscopic outcomes	Remission		Recurrence
Mean quartile sum score	14.98		16.41
P value		0.057	
Mean *n* positive markers	3.03		3.45
P value		0.115	
18-month serology for 18-month endoscopic outcomes	Remission		Recurrence
Mean quartile sum score	14.41		15.69
P value		0.094	
Mean *n* positive markers	2.75		3.24
P value		0.072	

likely to have endoscopic recurrence at 18 months than patients who were negative for all antibodies (82% *vs* 18%, $P = 0.034$; OR 6.4, 95% CI 1.16–34.9). When patients negative for all antibacterial antibodies were compared with patients positive for one or more antibacterial antibodies, patients who were negative were less likely to have disease recurrence at 18 months (11% *vs* 47%, $P = 0.004$).

There were no significant differences in the mean quartile sum score at baseline and the presence or absence of endoscopic recurrence at 6 or 18 months (Table 2; Fig. 3, panel a). The mean number of positive markers was associated with 18 month endoscopic outcome when measured at baseline ([recurrence *vs* remission; 3.8 *vs* 3.18, $P = 0.038$] [Table 2; Fig. 3, panel b]). When tested at 18 months, the number of positive antibodies was higher in patients with endoscopic recurrence compared with those in remission, although this was not significant (recurrence *vs* remission: 3.24 *vs* 2.75, $P = 0.07$).

The relationship between known clinical risk factors for recurrence and serological antibodies was examined. In the high risk patients, the mean baseline quartile sum score and number of positive markers was higher in those with endoscopic recurrence at 18 months (quartile sum score: 16.4 *vs* 14.6, $P = 0.045$, *n* positive: 3.86 *vs* 3.13, $P = 0.032$). In the low risk patients, neither the quartile sum score nor the mean number of positive markers was associated with disease recurrence at 18 months.

To evaluate the relationship between clinical risk factors, antibody profile, and disease recurrence at 18 months, the OR for the quartile sum score and number of positive markers were calculated using step-wise logistic regression (Table 3).

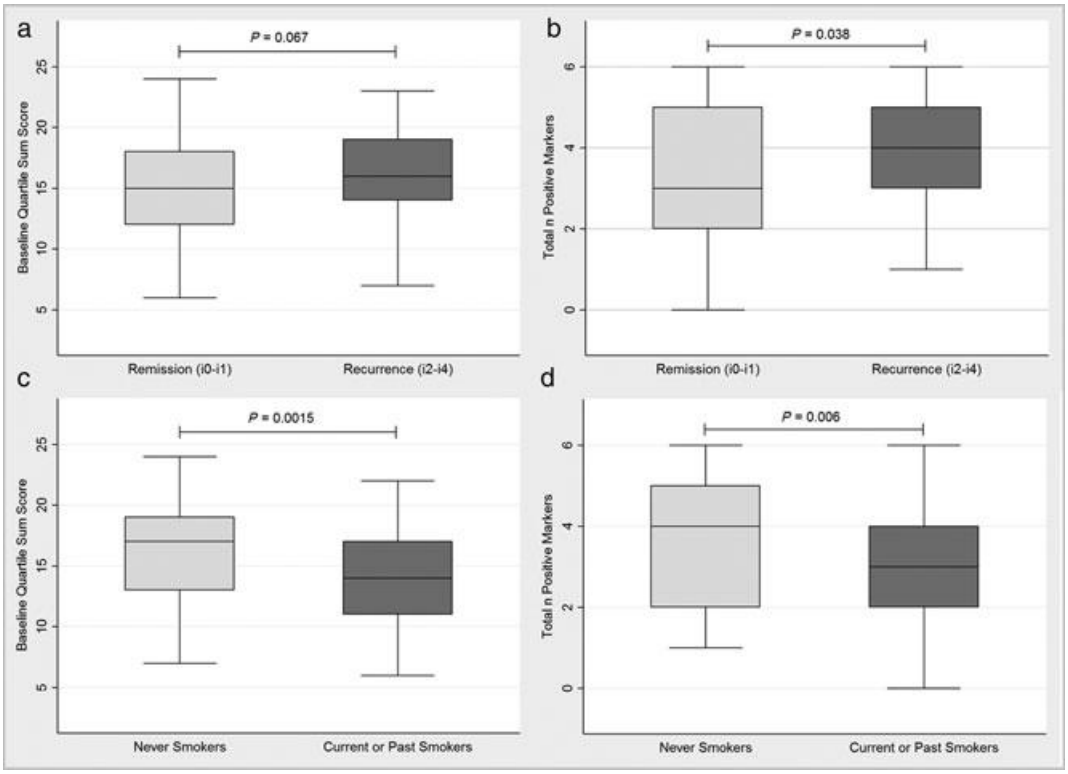

Figure 3 Panel (a) Baseline quartile sum score (quartiles: ASCA IgA and IgG, anti-OmpC, anti-CBir1, anti-A4-Fla2, anti-Fla-X) by 18-month endoscopic outcomes. (b) Baseline number of positive markers by 18-month endoscopic outcomes. (c) Baseline quartile sum score by smoking status. (d) Baseline number of positive markers by smoking status. All boxes denote median and inter quartile range.

Table 3 Adjusted odds ratios for baseline testing in relation to endoscopic outcome at 18 months, on step-wise logistic regression

	Total quartile sum score (range 6–24)				Total number of positive antibodies (range 0–6)			
	Adjusted OR	95% CI		*P* value	Adjusted OR	95% CI		*P* value
				Adjusted for smoking only				
Baseline	1.12	1	1.2	0.03	1.36	1.1	1.8	0.018
				Adjusted for all clinical risk factors (smoking, previous surgery, perforating disease)				
Baseline	1.13	1	1.3	0.02	1.40	1.1	1.8	0.013
				Adjusted for all clinical risk factors and pANCA status				
Baseline	1.13	1	1.3	0.02	1.40	1	1.8	0.013

CI, confidence interval; OR, odds ratio.

Quartile sum score at baseline was significant when adjusted for smoking (OR 1.12, 95% CI 1.0–1.2, $P = 0.03$). When adjusted for the clinical risk factors (smoking, perforating disease, and previous surgery) and pANCA, the OR was similar (OR 1.13, 95% CI 1.0–1.3, $P = 0.02$). The number of positive antibodies (range 0–6) measured at baseline was associated with disease recurrence at 18 months, after adjustment for smoking status (OR 1.36, 95% CI 1.1–1.8, $P = 0.018$).

The total baseline quartile sum score (but not number of positive markers) was significantly greater in patients with severe recurrence (Rutgeert's i3–i4) than in patients without severe recurrence at 18 months, when adjusted for clinical risk factors (OR 1.16, 95% CI 1.01–1.34, $P = 0.039$) and additionally adjusted for pANCA status (OR 1.17, 95% CI 1.01–1.36, $P = 0.034$).

Sensitivity and specificity. For the prediction of recurrence at 6 and 18 months, the total baseline quartile sum score AUROC was 0.50 and 0.60, respectively, and the number of positive markers AUROC was 0.51 and 0.59, respectively. The AUROC

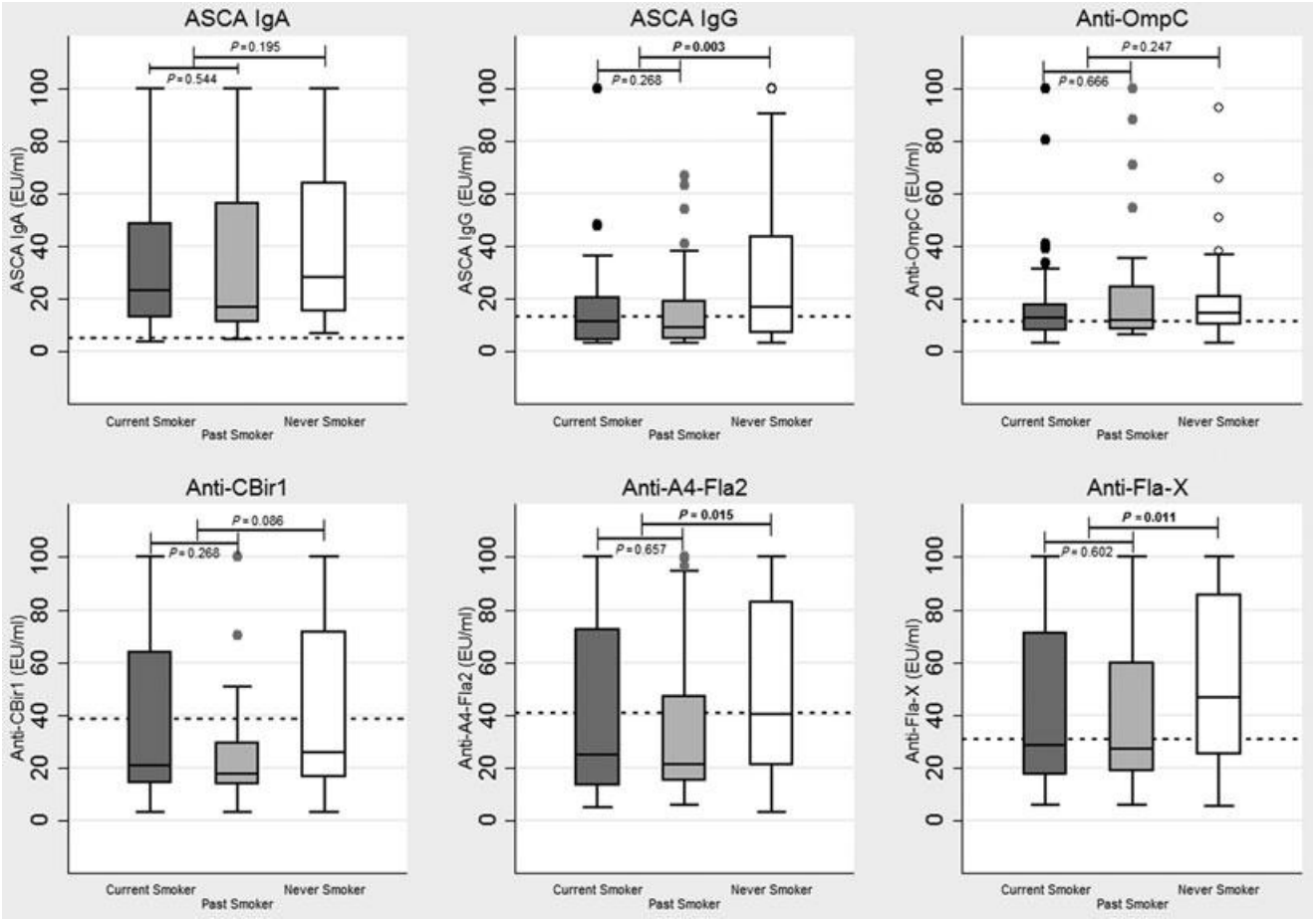

Figure 4 Baseline values for individual antibodies, stratified by smoking status. Dashed line represents reference range cut off. *P* values are for current *versus* past smokers, and current/past smokers combined *versus* never smokers. All boxes denote median and inter quartile range.

curves for the individual markers at baseline are shown in Figure S1.

Other assessments of clinical and disease recurrence

Crohn's disease activity index and faecal calprotectin. No antibody titers correlated significantly with subsequent Crohn's disease activity index measurements or faecal calprotectin at any timepoint.

Smoking. Patients who had never smoked were more likely than current or past smokers to be ASCA IgG positive (56% *vs* 40%, $P = 0.05$) and anti-Fla-X positive (67% *vs* 47%, $P = 0.01$) at baseline. Individual antibody levels stratified based on smoking status are compared in Figure 4.

The relationship between smoking and antibody presence or titer was assessed by comparing baseline antibody results between current ($n = 52$) *versus* never ($n = 76$) and past ($n = 41$) smokers combined (mean quartile sum score: 14.4 *vs* 15.3 [$P = 0.184$]; mean number of positive markers 3.2 *vs* 3.4 [$P = 0.432$]).

Comparing current and past smokers with patients who had never smoked revealed a significantly lower quartile sum score (14.1 *vs* 16.2 [$P = 0.0015$] [Fig. 3, panel c]) and lower number of positive markers (3.0 *vs* 3.7 [$P = 0.006$] [Fig. 3, panel d]).

Discussion

The accuracy of serologic antibodies for identifying patients at increased risk of Crohn's disease postoperative disease recurrence, and identifying patients with recurrence, has not been prospectively addressed in a large cohort. Anti-Fla-X had the greatest value in relation to predicting subsequent recurrence. Recurrence at 18 months was associated with anti-Fla-X positivity at all time points although significantly so only at baseline and at 12 months postoperatively.

Baseline positivity for all four bacterial antibodies (anti-CBir1, anti-OmpC, anti-A4-Fla2, and anti-Fla-X) was also more likely to have early recurrence than those negative for all antibodies. Seventeen percent of patients were positive for all four antibodies, potentially identifying a group of patients who have a higher risk of recurrence separate to the clinical risk factors. Adjustment for clinical risk factors improved the predictive ability of antibody testing.

Two antibodies, anti-A4-Fla2 and anti-OmpC, related to previous surgery and may be regarded as markers for risk of reoperation. Anti-A4-Fla2 was negatively associated with the risk of surgery, and anti-OmpC was positively associated with two or more previous operations. Neither of these two antibodies was predictive for, or associated with, recurrent disease. The A4-Fla2 and Fla-X flagellins have been mapped to the family *Lachnospiraceae,* an anaerobic non-pathogenic bacterial family within the Firmicutes phylum.[6,25] OmpC is an *E. coli* outer membrane protein that represents an immune response to the *Enterobacteriaceae* family.[32]

In relation to increased risk of severe recurrence (≥ Rutgeerts i3), the total baseline quartile sum score (but not number of positive markers) was significantly greater in patients with, than patients without, severe recurrence at 18 months.

The presence and magnitude of many serological antibodies varies little according to disease activity,[31] treatment,[33,34] and surgery.[17] ASCA IgA and IgG levels do not vary in relation to surgery or endoscopic recurrence.[17] Our results demonstrate that mean titers of individual antibodies vary significantly (Fig. 2), but the magnitude of change is small and unlikely to be pathophysiologically relevant. Measurement of changes in titer of these antibodies over time is therefore not clinically useful.

We have demonstrated lower humoral immune activation in current and past smokers across multiple antibodies. This has been shown previously in periodontal disease,[35] systemic lupus erythematosus,[36] healthy subjects,[37,38] and in a cohort of 113 Crohn's disease patients using only a single antibody (ASCA).[39] That past smokers cluster with current smokers in the presence and magnitude of these antibodies suggests that immune downregulation may not resolve on cessation of smoking; this remains to be tested with stricter smoking criteria. The lower titer of antibodies in past or current smokers compared with "never" smokers indicates that the increased risk of recurrence associated with smoking is mediated through a separate mechanism to that reflected in antibody production. When interpreting antibody levels, serological testing should be interpreted in the light of smoking status.

The pathophysiology of antibody development to luminal antigens is currently unclear. Antibody levels do not decline after surgery, suggesting that an immune change, such as one related to the genetic background or gut microbiome, is more important than disease burden. Mutations in microbial pattern recognition receptor genes (NOD2) and autophagy genes (ATG16L) have been linked with the development of ASCA, and IGRM mutations with antibody responses to CBir1 flagellins.[40] Anti-OmpC shows high concordance (Intraclass correlation coefficient of 0.80) between monozygotic twin pairs discordant for CD implying a possible genetic predisposition for antibody development.[41] Because the CBir1 and Fla-X antigens have 84% amino acid sequence overlap,[26] and A4-Fla2 and Fla-X also overlap significantly,[25] a susceptible genetic background may influence the immune reactivity to other closely related flagellin proteins.

The strengths of this study include strict phenotyping and endoscopic assessment rather than outcomes based on clinical recurrence or imaging, and defined time points. This study used established reference ranges to define antibody positivity or negativity.

Our patient population had a high ASCA positivity rate. This may relate to the sensitivity of the IgA and IgG assays used or may reflect the high proportion of patients with complex disease.[5,16,17,42]

In summary, serologic antibodies may add value in the prediction of postoperative disease recurrence. Smoking status should be considered in relation to serologic testing. Their role in providing pathophysiological insights remains to be explored. Patients who demonstrate positivity for multiple serologic antibodies remain at risk of a more complex disease course and a greater need for surgical intervention.[6–8,12,15,42] These serologic markers may identify patients who would benefit from more aggressive monitoring and therapy of their disease generally, and following intestinal resection.

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Conference presentations

This work was presented in part at the European Crohn's and Colitis Organization Congress 2015, Barcelona, Spain, and Digestive Diseases Week 2015, Washington D.C., USA.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. AUROC curves for baseline serological prediction of endoscopic recurrence at 18 months post operatively for each antimicrobial marker.

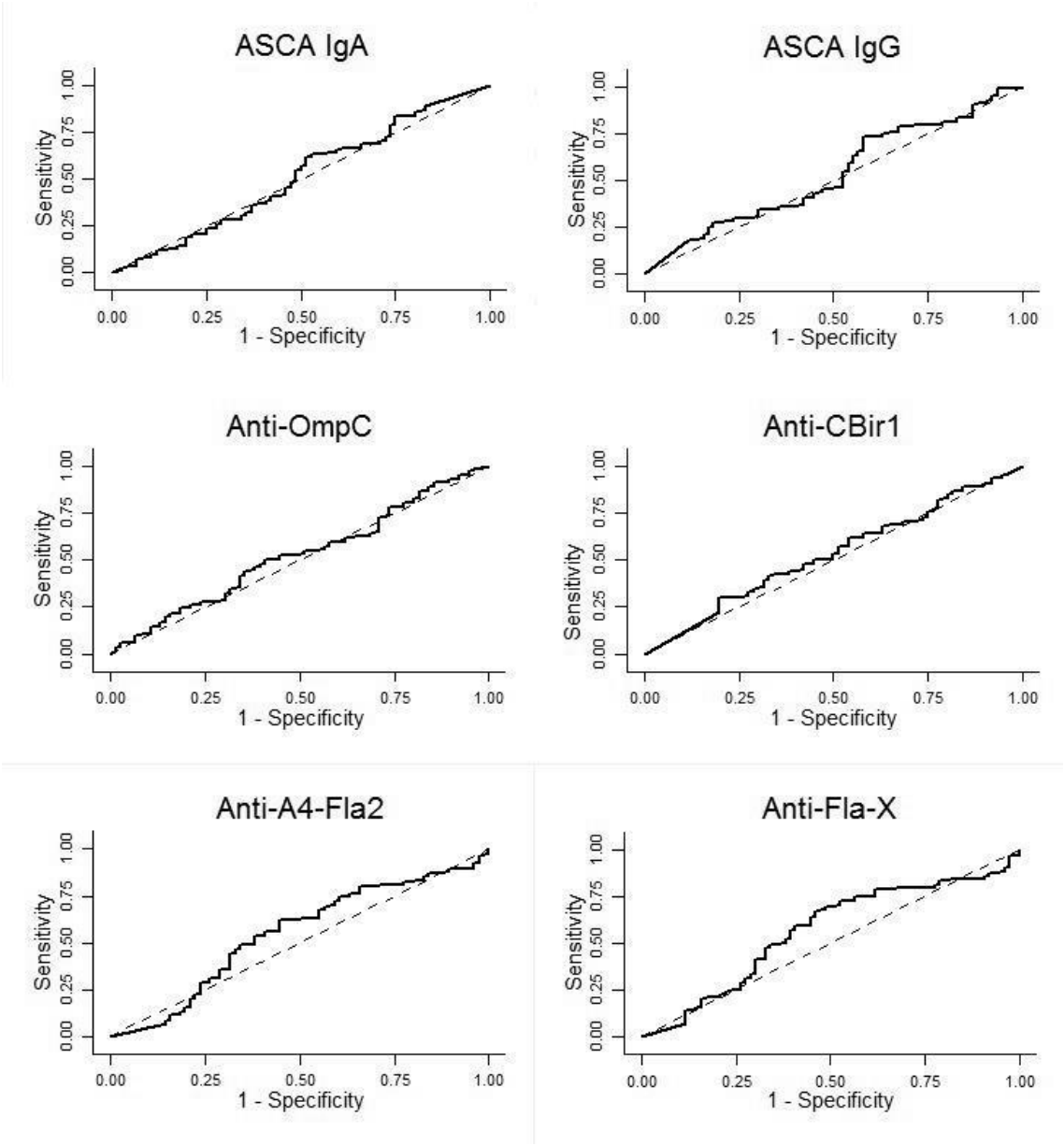

Figure 3.1 Supplementary Figure 1 – AUROC curves for individual markers at baseline for prediction of post-operative recurrence at 18 months

4 The Faecal Microbiome in Post-Operative Crohn's Disease

4.1 Introduction

Crohn's disease is a chronic, inflammatory bowel condition that is rapidly increasing in prevalence around the world, especially in western countries.[1] Crohn's disease is believed to result from perturbation of a number of interacting factors including genetic background, host immune responses, the gut microbiome and environmental factors. Up to 80% of patients with Crohn's disease (CD) require intestinal resection at some stage following diagnosis, and 75% of patients develop post-operative recurrence, which requires further surgery.[3, 4, 27] While the contribution of the microbiome to Crohn's disease has been investigated in a number of cohorts[102, 105, 171], a large study of the faecal microbiota following a bowel resection for active disease has not been undertaken.

Two bacterial communities exist in the bowel, the mucosally associated microbiota and the faecal microbiome (the luminal stream). There are differences in the abundance of bacterial families in these ecosystems, even within healthy individuals. In the faecal microbiome, overall diversity of species (as measured by both α and β diversity metrics) is greater than at the mucosal surface, while the mucosally associated microbiome is enriched for the *Proteobacteria*, *Firmicutes* and *Bacteroidetes* phyla.[132] Metagenomic studies of the gut microbiome from Crohn's disease patients have shown a pattern of dysbiosis that is generally consistent, including a decrease in *Firmicutes* (presence of, and diversity within) and an increase in *Gammaproteobacteria* (particularly *Enterobacteriaceae*).[90, 148]

Post-operative dysbiosis has been characterised by only a few studies, although general trends have emerged such as a global reduction in the abundance of *Faecalibacterium prausnitzii* in patients with disease recurrence after surgery.[112, 154, 170] However, analysis of the post-operative microbiome is perturbed by the administration of antibiotics, host inflammation and other post-operative therapies. As such, it would be helpful to characterise the features of the luminal communities associated with both post-operative remission and recurrence in this specific patient population. This may allow non-invasive identification of patients with, or at risk of recurrence even in the presence of ecologic modifying factors such as antibiotics and disease therapies.

We have previously assessed changes in the mucosally associated microbiota in a subset of patients from the Post-operative Crohn's Endoscopic Recurrence (POCER) study, which showed a lower abundance of *Faecalibacterium prausnitzii* (<0.1% abundance; OR 14 [1.7-110], P = 0.013) and a detectable abundance of *Proteus* species (from the *Enterobacteriaceae* family) within the ileum (OR 13 [1.1 -150], P = 0.039) was associated with endoscopic recurrence.[154]

In this study, we wish to characterise the overall differences and patterns in the faecal microbiota that may explain the risk of disease recurrence in these patients, followed up to 18 months from the time of surgery using objective measures (endoscopic assessment). Patients enrolled in this study were required to have all macroscopic disease removed at surgery, providing a "clean slate" from which to investigate the development of recurrent Crohn's disease.

The contribution of the faecal microbiome to the development of post-operative recurrence has not been addressed in a large prospective patient population. This is the first large-scale use of high throughput sequencing of faecal samples in the context of post-operative disease recurrence in CD.

4.2 Methods

4.2.1 Subjects and Ethical Approvals

One hundred and thirty-six individuals provided 314 faecal samples while participating in the Post-Operative Crohn's Endoscopic Recurrence (POCER) Study (Clinical Trial Registration: NCT00989560).[6] The POCER Study was a multicentre, randomised controlled trial undertaken at 17 clinical sites, addressing the utility of defined surveillance and treatment strategies for prevention of post-operative Crohn's disease recurrence in high and low risk patients. As part of the approved protocol (approved by the Human Research Ethics Committees of St Vincent's Public Hospital, Melbourne, Australia and all other participating hospitals) faecal samples were collected peri-operatively (within 2 weeks of surgery), and at six, 12 and 18 months post operatively.

4.2.2 Clinical Covariates

The POCER study design is outlined in Figure 2.1. Phenotypic characteristics of patients were recorded at baseline using the Montreal Classification.[10] Medication use, the Crohn's Disease Activity Index[24] and symptoms were recorded at each major visit. Patients in the active care arm underwent a colonoscopy at six months with all patients

undergoing a (further) colonoscopy at 18 months post operatively where patients were assessed for endoscopic disease recurrence. Body mass index was computed at baseline for all patients to exclude the effect of body weight on the microbiome. All patients were prescribed 3 months of metronidazole (400mg b.d.), 77% (100/130 patients) of patients tolerated the dose and adherence to this dose was used as an adjusting factor in all analyses. Demographic information for this patient cohort is shown in Table 4.1.

4.2.3 Assessment of Endoscopic Disease Recurrence

Primary assessment of anastomotic and ileal disease recurrence was performed using the Rutgeerts score[3] by the gastroenterologist. A consensus score was determined on review of photographs by two principal investigators (PDC, MAK).

4.2.4 Faecal Sample Collection and DNA extraction

Faecal samples were collected by patients within three days of the visit, prior to the administration of any bowel preparation and frozen initially at -20°C for transport. Samples were then stored at -80°C until analysis.

4.2.5 PCR for 16s ribosomal gene amplification

The bacterial 16S V2 region was PCR amplified with universal bacterial 16s primers F101, and 342R,[211, 212] and barcoded with Illumina index/adaptors using the Expand High Fidelity PCR kit (Roche). PCR products were purified with the Wizard SV gel and PCR clean-up system (Promega, Madison, USA). The DNA was quantitated using and sequenced by the Australian Genome Research Facility (AGRF) using the Illumina Miseq platform producing 250bp paired end reads from 500 cycles.

4.2.6 Bioinformatic and Statistical Analysis

The metagenomic analysis of bacterial 16S sequencing reads was performed using the QIIME (Quantitative Insights into Microbial Ecology, Version 1.91)[208] program, specifically designed for the metagenomic analysis of large 16S datasets.

Briefly, reads were trimmed to 200bp using FASTX-Toolkit Version 0.0.14 (http://hannonlab.cshl.edu/fastx_toolkit/), and merged using FLASH Version 1.2.11.[207] Within QIIME, reads were quality filtered (allowed n's = 1), a closed reference pick was performed for QC and sample selection. Technical issues affected the merge percentage of 23 samples from 2/4 sequencing plates, in this case the R1 read was trimmed, but not merged. A further quality threshold was set, including only samples with a merge percentage of >90% and at least 15,000 reads per sample prior to

merging, leaving 288 of 314 samples from 130 patients for analysis. Chimeric reads were identified using the USEARCH Version 6.1[213], referenced against the rdp_Gold database and filtered out using QIIME. 1,369,078 (6.54%) reads were removed from the quality filtered reads, leaving 23,577,525 reads for analysis. These reads were assigned to OTU's using the subsampled open reference method and the Greengenes 97% OTU reference set, version 13_8[210], to remove reads with less than 60% sequence identity, with 22,491,263 reads remaining (av. 78,095 per sample, range 10,701-665,671). The final read distribution for this dataset is shown in Figure 4.1.

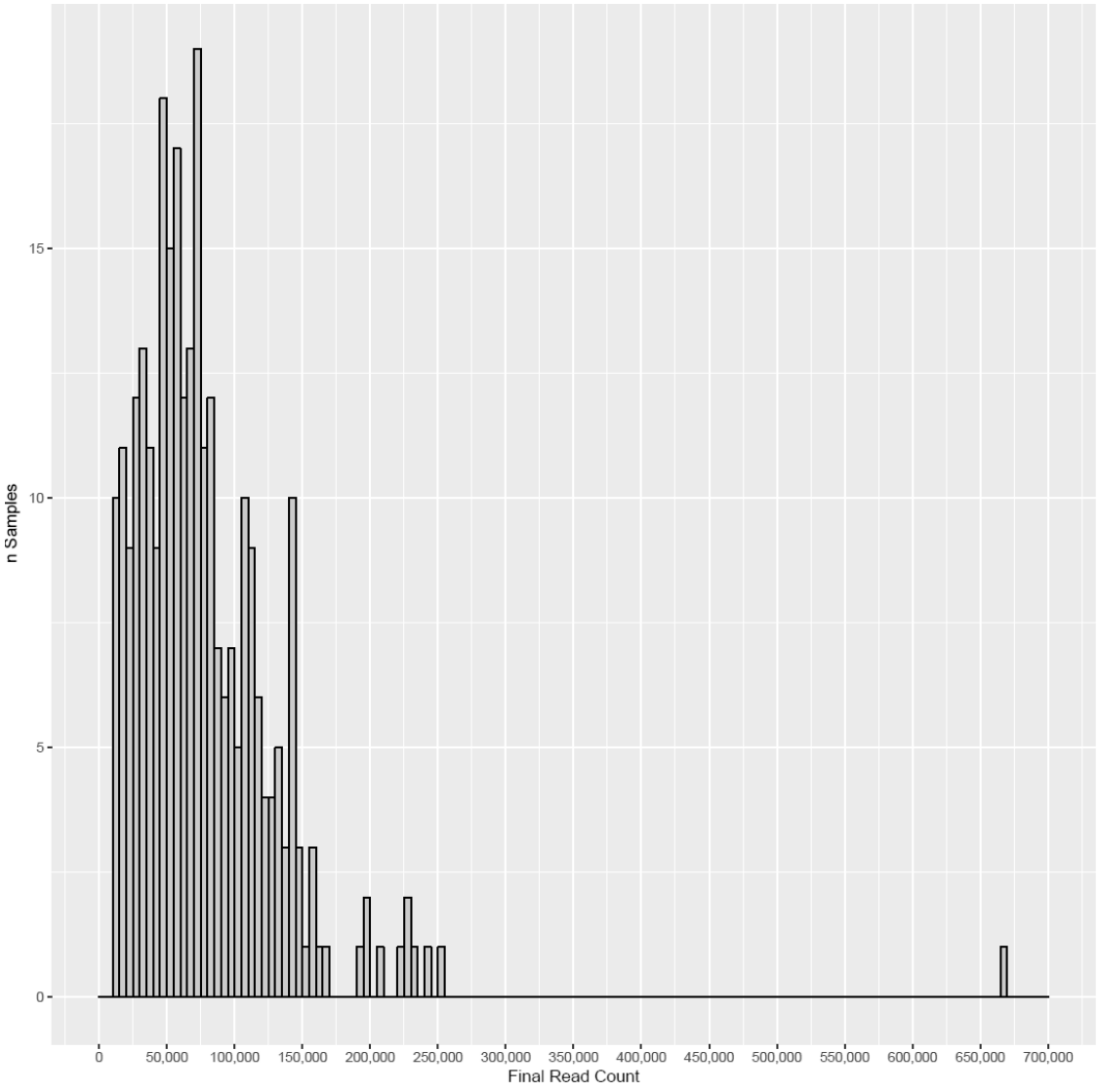

Figure 4.1 Distribution of Read Counts for all samples following open reference picking

Samples were sub sampled and rarefied to 10,000 reads per sample prior to diversity analysis. Alpha diversity was calculated using number of OTU's, Shannon's Diversity

Index (SDI) and Chao 1 Diversity Index. The Wilcoxon rank sum test was used to compare alpha diversity metrics, the Kruskal-Wallis rank sum test was used where there were more than two groups. Beta diversity was calculated using a non-parametric multivariate ANOVA on both the weighted and unweighted UniFrac distance matrix (adonis package) in R.[97, 214, 215]

Differential abundance at family level was performed on cumulative sum scaled (CSS normalisation) OTU tables by zero-inflated Gaussian (ZIG) modelling, using the MetagenomeSeq package in R.[215, 216] This normalisation method (scaling factor 0.61) is designed to account for possible biases from uneven sampling depths, while the ZIG distribution mixture model reduces bias from undersampling and allows correction for possible confounders.[216] Taxa were excluded if not present in at least 20% of samples for each comparison, and additional adjustments were made for number of comparisons (FDR). All taxonomic assessments were corrected for age, gender, smoking status (except where smoking was the primary variable), metronidazole tolerance and body mass index. Additional variables (sequencing plate and hospital of origin) were also included as additional correcting factors to exclude experimental variation. The change of abundance is given as a positive (increase) or negative (decrease) log2 fold change (LFC), with significance (≤0.05) reported as both unadjusted and false-discovery rate adjusted (FDR) P values.

4.2.7 Cluster Analysis

Hierarchical cluster analysis (1-Pearson's correlation, Ward's minimum variance, hclust function) was performed on the relative abundance for all samples regardless of timepoint, collapsed at family level (average relative abundance >10% across all samples). Silhouette analysis (using the Cluster package in R[215]) was performed to obtain the optimal cluster number. A silhouette value is a mathematical representation of the tightness vs separation of each object within assigned cluster and is used to determine the "appropriateness" of the number of clusters assigned.[217] A value is calculated for each cluster identified. This value, from 0, (representing total separation) to 1.0 (1.0 being total similarity) allows assessment of the overall quality and validity of the number of clusters chosen. [217]

Each cluster was assessed for outcome at 18 months using generalised estimating equations (GEE) adjusted for sample time point, smoking status, BMI, antibiotic use, age, gender, sequencing plate and hospital of origin.

Following identification of significant clusters at family level, the three major clusters (*Lachnospiraceae*, *Bacteroidaceae* and *Enterobacteriaceae*) were sub-clustered at OTU level (relative abundance of at least 0.1% average, 10% abundance in at least one sample), and the sub-clusters were compared to all other samples for endoscopic outcomes at 18 months. The sub-clusters within each family were then compared against all other samples for outcome at 18 months using GEE adjusted for sample time point, smoking status, BMI, antibiotic use, age, gender, sequencing plate and hospital of origin.

4.3 Results

Of the 288 samples that were analysed from 130 patients, 61 samples were obtained peri-operatively (baseline), 86 at six months, 80 at 12 months and 61 at 18 months. Demographics and baseline characteristics of the cohort are listed in Table 4.1. Matched colonoscopic outcomes were available for 60/86 six month samples, of which 21 (35%) had endoscopic recurrence, and for all of the 18 month samples, of which 20 (33%) had endoscopic recurrence. The dominant phyla at baseline were *Firmicutes* (78.9%), *Proteobacteria* (10.0%), *Bacteroidetes* (6.7%), *Actinobacteria* (3.0%), *Verrucomicrobia* (1.1%) and *Fusobacteria* (0.3%).

Demographics	n	%
n [male]	57	43.85
Age, median	36	
Interquartile range [IQR]	(26-46.75)	
Active smoker	38	29.23
Body mass index, median	23.58	
Interquartile range [IQR]	(20.59-28.11)	
Age at diagnosis:		
A1 ≤ 16 years	16	12.31
A2 17–40	94	72.31
A3 > 40	20	15.38
Duration of Crohn's disease (years):		
Median Duration	10	
Interquartile range [IQR]	(4-15)	
> = 10 years	66	50.77
Disease location at surgery:		
L1 Ileum only	69	53.08
L2 Colon only	9	6.92
L3 Ileum and colon	52	40.00
L4 Upper GI	7	5.38
Disease phenotype at surgery:		
B1 Inflammatory	12	9.23
B2 Stricturing	49	37.69
B3 Penetrating	69	53.08
P Perianal Disease	16	12.31
Indication for surgery:		
Failure of drug therapy	29	22.31
Obstruction	38	29.23
Perforation	63	48.46
Type of surgical resection:		
Ileocolic Resection	102	78.46
Small Bowel Resection	7	5.38
Subtotal Colectomy	6	4.62
Simultaneous ileocolic resection and colectomy	4	3.08
Simultaneous ileocolic and isolated small bowel resection	11	8.46
Number of previous surgical resection:		
0	91	70.00
1	28	21.54
2	6	4.62
3 or more	5	3.85
Immediate postoperative baseline drug therapy:		
Metronidazole alone	23	17.69
Thiopurine	72	55.38
Adalimumab	35	26.92
6-month endoscopic outcomes (n = 92):		
Remission (Rutgeerts i0-i1)	60	65.22
Recurrence (Rutgeerts i2-i4)	32	34.78
18-month endoscopic outcomes (n = 105):		
Remission (Rutgeerts i0-i1)	60	57.14
Recurrence (Rutgeerts i2-i4)	45	42.86

Table 4.1 Demographics of the Microbiota Cohort

4.3.1 Alpha Diversity

Within-sample (alpha) diversity results are summarised in Table 4.2. Alpha diversity at baseline differed significantly according to: (1) Disease location. Ileal disease was associated with a higher Shannon Diversity Index (SDI) SDI than ileo-colonic or colonic disease; (2) Prior surgery. Prior surgery was associated with lower diversity (P = 0.050).

Within all patients over time diversity increased from the time of surgery to 18 months post-operatively (Figure 2; median SDI 5.23 vs 6.10; P = 0.048). The greatest increase in diversity occurred between 12 and 18 months post-operatively (median SDI 5.26 vs 6.10; P = 0.009), with no significant difference between baseline and 12 months.

At six months there was no significant differences in alpha diversity seen between patients that took or did not take the prescribed dose (median SDI 5.24 vs 5.14; P = 0.679).

Alpha diversity at 6 months in patients with subsequent endoscopic recurrence at 18 months was lower than those with endoscopic remission at 18 months (Table 4.2, median SDI 4.44 vs 5.69; P = 0.041).

At 18 months alpha diversity did not differ between recurrence and remission (Table 4.2, median SDI 5.94 vs 6.10; P = 0.186). Further, there were no differences when comparing extremes in outcome (mucosal normality - i0 vs. severe recurrence - i3/4) between baseline and 6 or 18 months, and within time points.

Alpha Diversity	**Group 1**	**n**	Median SDI	IQR	**Group 2**	**n**	Median SDI	IQR	**Group 3**	**n**	Median SDI	IQR	***P* Value**
Baseline Characteristics													
Smoking	Current Smokers	13	4.75	4.16-6.38	Past and Never Smokers	48	5.24	3.30-6.59	-	-	-	-	0.855
	Current and Past Smokers	31	5.26	3.32-6.45	Never Smokers	30	5.20	3.48-6.93	-	-	-	-	0.802
	Current smokers	13	4.75	4.16-6.38	Past Smokers	18	5.54	3.03-6.46	Never Smoker	30	5.20	3.475-6.934	0.899
Baseline Disease Phenotype													
Disease Location	**Ileal**	**30**	**6.10**	**4.35-7.19**	**Colonic**	**4**	**4.13**	**1.74-6.79**	**Ileocolonic**	**27**	**4.73**	**2.358-5.990**	**0.032**
Disease Behaviour	Inflammatory	8	5.51	5.04-6.52	Stricturing	23	5.17	3.56-6.42	Penetrating	30	5.10	3.131-6.616	0.693
Prior Surgery	**No Prior Surgery**	**42**	**5.79**	**3.80-6.88**	**Prior Surgery (>1 resection)**	**19**	**4.73**	**2.80-5.76**	-	-	-	-	**0.050**

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Endoscopic Remission versus Recurrence	**Group 1**	**n**	Median SDI	IQR	**Group 2**	**n**	Median SDI	IQR					
6 Month Outcomes													
Baseline	Remission	30	5.14	3.43-6.52	Recurrence	16	6.20	4.31-6.66	_	-	-	-	0.515
	Normality (i0)	11	4.59	2.76-6.12	Severe Recurrence (i3/i4)	9	4.73	1.92-5.21	_	-	-	-	0.710
at 6 months	Remission	40	5.27	3.19-5.92	Recurrence	22	4.98	3.74-6.66	_	-	-	-	0.256
	Normality (i0)	20	3.89	2.64-5.96	Severe Recurrence (i3/i4)	9	5.40	3.14-6.29	_	-	-	-	0.777
18 Month Outcomes													
Baseline	Remission	24	5.46	3.50-7.58	Recurrence	25	4.98	3.45-6.21	_	-	-	-	0.699
	Normality (i0)	8	3.45	2.09-4.54	Severe Recurrence (i3/i4)	10	4.74	2.08-6.49	_	-	-	-	0.408
at 6 Months	**Remission**	**42**	**5.69**	**4.08-6.36**	**Recurrence**	**30**	**4.44**	**3.14-5.67**	_	-	-	-	**0.041**
	Normality (i0)	16	5.85	4.25-6.42	Severe Recurrence (i3/i4)	13	5.40	2.50-5.70	_	-	-	-	0.092
at 18 months	Remission	41	6.10	5.13-7.02	Recurrence	20	5.94	3.81-6.63	_	-	-	-	0.186
	Normality (i0)	16	6.54	5.21-7.14	Severe Recurrence (i3/i4)	5	6.16	3.15-6.76	_	-	-	-	0.445

Table 4.2 Alpha Diversity Outcomes by baseline characteristics, phenotype and endoscopic disease status (SDI, Shannon's Diversity Index)

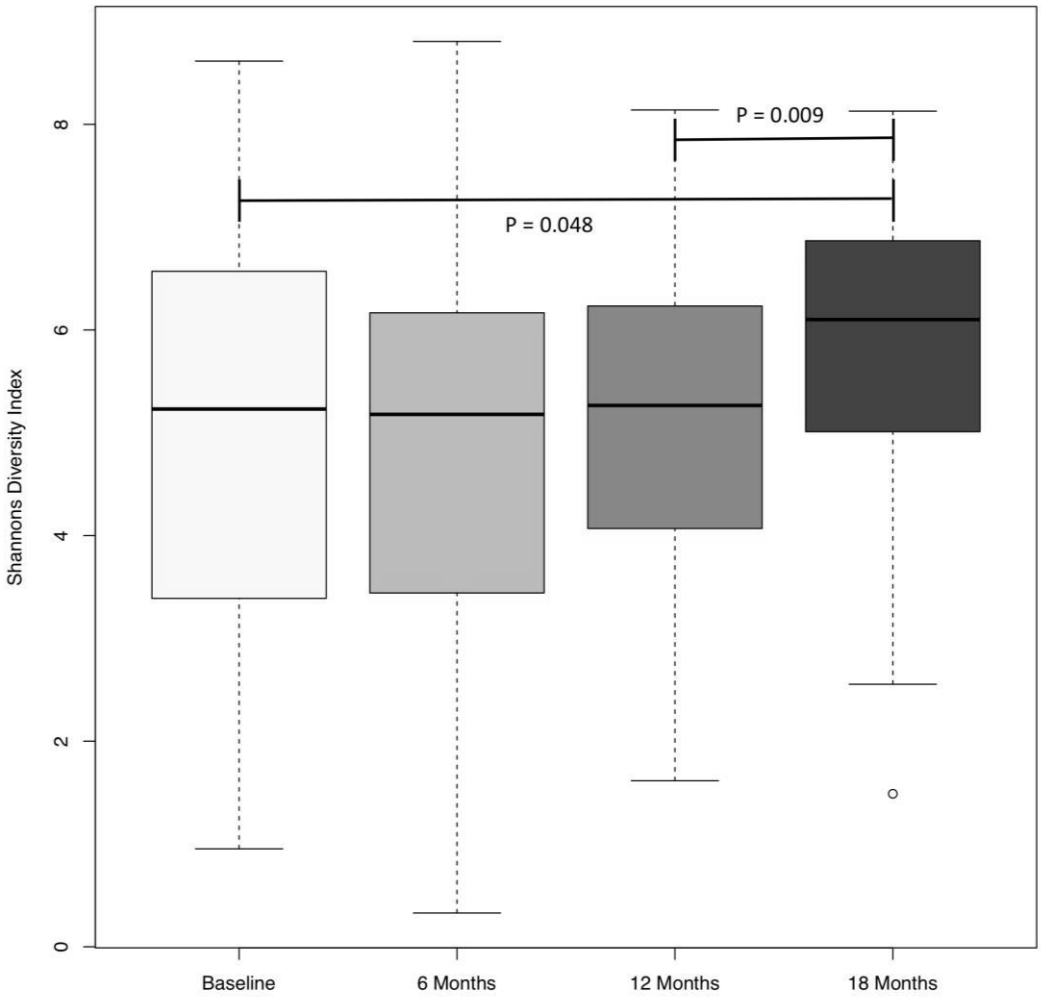

Figure 4.2 Alpha Diversity over time as measured by the Shannon's Diversity index, with Wilcoxon Rank sum test for significance

4.3.2 Beta Diversity

Community composition (both presence/absence – unweighted UniFrac and abundance – weighted UniFrac) changed significantly between all timepoints after surgery although the effect sizes were small (all patients, baseline versus 18 months; unweighted R^2 0.021, FDR P = 0.008 and weighted R^2 0.069, FDR P = 0.013 respectively). The six-month samples were assessed for differences in beta-diversity based on tolerance of metronidazole, but this was not significant. However as nearly all patients trialed metronidazole (but did not adhere completely to this treatment), this may be a confounding factor in subsequent analysis. This reflects the high proportion of Crohn's disease patients treated with antibiotics as part of standard treatment practices.

When baseline characteristics were addressed, there were differences in composition based on smoking status but only on unweighted UniFrac when analysed with the Adonis multivariate analysis method. This implies that once abundances are taken into account there are no differences in diversity based on smoking (**Table 4.3**; all samples; current versus past versus never smokers, unweighted, FDR *P* = 0.009) or disease location (all samples; Montreal classification, unweighted, FDR *P* = 0.020).

Overall bacterial composition differed in samples taken at 18 months, when recurrence and remission at 18 months was assessed (weighted UniFrac; *P* =0.008), but this was not significant after adjustment (FDR *P* = 0.069) and the effect size is small. Further beta diversity outcomes are summarised in Table 4.3.

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Beta Diversity	Weighted Unifrac			Unweighted Unifrac		
	R^2	*P* Value	FDR *P* Value	R^2	*P* Value	FDR *P* Value
Baseline Characteristics						
By Smoking Status (3 group)						
All Samples	0.003	0.513	0.785	0.006	**0.001**	**0.009**
Baseline	0.012	0.644	0.800	0.017	0.395	0.594
6 Months	0.015	0.27	0.598	0.017	**0.009**	**0.029**
18 Months	0.008	0.811	0.879	0.017	0.398	0.594
***By Disease Location* (Ileal, Ileocolonic and Colonic)**						
All Samples	0.005	0.106	0.459	0.005	**0.003**	**0.020**
Baseline	**0.041**	**0.025**	0.163	0.025	**0.008**	**0.029**
6 Months	0.007	0.721	0.852	0.011	0.757	0.820
18 Months	0.006	0.885	0.895	0.019	0.113	0.245
Remission vs. Recurrence						
Baseline samples for 6 month outcomes	0.013	0.792	0.879	0.019	0.943	0.957
6 Month samples for 6 month outcomes	0.020	0.264	0.598	0.015	0.684	0.789
Baseline samples for 18 month outcomes	0.016	0.595	0.800	0.019	0.698	0.789
6 Month samples for 18 month outcomes	0.014	0.372	0.722	0.016	0.091	0.237
18 Month samples for 18 month outcomes	**0.074**	**0.008**	0.069	0.019	0.111	0.245
Mucosal Normality (i0) vs. Severe Recurrence (i3/4) at 6 months						
All Samples	0.013	0.389	0.722	0.014	0.474	0.649
Baseline	0.085	0.212	0.598	0.060	0.553	0.719
6 Months	0.041	0.446	0.773	0.046	0.208	0.386
Mucosal Normality (i0) vs. Severe Recurrence (i3/4) at 18 months						
All Samples	0.013	0.228	0.598	0.015	**0.006**	**0.026**
Baseline	0.037	0.646	0.800	0.063	0.25	0.433
6 Months	0.031	0.561	0.800	0.034	0.632	0.782
18 Months	0.060	0.276	0.598	0.053	0.204	0.386

Table 4.3 Beta Diversity Outcomes

Weighted and unweighted UniFrac Index, with significance using the Adonis method; a permutational multivariate analysis of variance (MANOVA) performed on distance matrices

4.3.3 Taxonomic Changes associated with Disease Recurrence

The differential abundance of taxa at family level for remission v recurrence are shown in Table 4.4. All samples were assessed for outcomes at six and 18 months, as well as the within time-point samples. A positive log fold change represents a relative increase in patients with disease recurrence at 18 months. *Corynebacteriaceae* (phylum *Actinobacteria*) was significant when all samples were considered for both timepoints, but differing in directionality (LFC at six months; -0.57, FDR *P* = 0.0005, LFC at 18 months; 0.84, FDR *P* = <0.0001). *Carnobacteriaceae* (phylum *Firmicutes*) was also reduced in samples with recurrence at six and 18 months (six months: LFC -0.94, unadjusted *P* = 0.11, 18 months: LFC -0.74, FDR *P* = 0.045). Overall, the taxonomic changes across time were predominantly within the *Firmicutes* phylum (*Firmicutes* 19/27 families; across all significant comparisons; Table 4.4), but the direction of the LFC was not consistent

Family	n samples positive Remission (av. norm counts)	n samples positive Recurrence (av. norm counts)	Log2 Fold Change	95% CI		*P* Value	FDR Adjusted *P* Value	Taxonomy
All Samples for 18 Month Outcome								
f Corynebacteriaceae	32 (26.87)	25 (57.45)	**0.84**	0.63	1.05	**<0.0001**	**<0.0001**	p Actinobacteria; c Actinobacteria; o Actinomycetales; f Corynebacteriaceae
f Christensenellaceae	41 (193.77)	14 (97.84)	**0.94**	0.52	1.35	**<0.0001**	**0.0005**	p Firmicutes; c Clostridia; o Clostridiales; f Christensenellaceae
f__Aerococcaceae	36 (61.06)	19 (112.73)	**0.41**	0.14	0.68	**0.004**	**0.043**	p Firmicutes; c Bacilli; o Lactobacillales; f Aerococcaceae
f__Eubacteriaceae	47 (295.01)	20 (56.73)	**-0.48**	-0.81	-0.15	**0.005**	**0.044**	p__Firmicutes; c__Clostridia; o__Clostridiales; f__Eubacteriaceae
f Enterococcaceae	84 (141242.76)	68 (416742.95)	**0.96**	0.11	1.82	**0.027**	0.184	p Firmicutes; c Bacilli; o Lactobacillales; f Enterococcaceae
f__	148 (80906.02)	106 (126946.98)	**-0.54**	-1.04	-0.04	**0.034**	0.193	p Firmicutes; c Clostridia; o Clostridiales; f
18 Month Samples for 18 Month Outcome								
f__Carnobacteriaceae	21 (36.34)	6 (6.48)	**-0.75**	-1.19	-0.31	**0.002**	0.052	p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Carnobacteriaceae
f Turicibacteraceae	14 (145.76)	5 (17.57)	**-1.24**	-2.31	-0.17	**0.026**	0.252	p Firmicutes; c Bacilli; o Turicibacterales; f Turicibacteraceae
f__	41 (20978.56)	20 (4893.25)	**-1.04**	-1.97	-0.11	**0.029**	0.252	p Firmicutes; c Clostridia; o Clostridiales; f
f__Actinomycetaceae	28 (111.72)	9 (11.56)	**-0.74**	-1.44	-0.04	**0.038**	0.252	p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Actinomycetaceae
f Porphyromonadaceae	33 (2280.39)	18 (16741.72)	**1.81**	0.01	3.61	**0.049**	0.252	p Bacteroidetes; c Bacteroidia; o Bacteroidales; f Porphyromonadaceae
f Eubacteriaceae	11 (22.36)	2 (3.73)	**-0.67**	-1.34	0.00	**0.050**	0.252	p Firmicutes; c Clostridia; o Clostridiales; f Eubacteriaceae
All Samples for 6 Month Outcome								
f__Corynebacteriaceae	33 (52.38)	18 (21.33)	**-0.57**	-0.81	-0.33	**<0.0001**	**0.0005**	p Actinobacteria; c Actinobacteria; o Actinomycetales; f Corynebacteriaceae
f Streptococcaceae	119 (171209)	65 (8847.71)	**-1.21**	-2.04	-0.39	**0.004**	0.070	p Firmicutes; c Bacilli; o Lactobacillales; f Streptococcaceae
f Prevotellaceae	116 (556761.82)	62 (21763.84)	**-1.11**	-1.98	-0.24	**0.013**	0.146	p Bacteroidetes; c Bacteroidia; o Bacteroidales; f Prevotellaceae
f__Veillonellaceae	131 (713566.72)	71 (200657.27)	**-1.30**	-2.37	-0.23	**0.018**	0.149	p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae
f__Erysipelotrichaceae	125 (12196.85)	68 (29433.57)	**0.92**	0.12	1.72	**0.025**	0.149	p__Firmicutes; c__Erysipelotrichi; o__Erysipelotrichales; f__Erysipelotrichaceae
f Gemellaceae	48 (368.7)	36 (205.18)	**-0.48**	-0.91	-0.06	**0.026**	0.149	p Firmicutes; c Bacilli; o Gemellales; f Gemellaceae
f__Peptostreptococcaceae	66 (2561.27)	35 (241.68)	**-0.64**	-1.28	-0.01	**0.047**	0.228	p Firmicutes; c Clostridia; o Clostridiales; f Peptostreptococcaceae

Continued over page

Family	n samples positive Remission (av. norm counts)	n samples positive Recurrence (av. norm counts)	Log2 Fold Change	95% CI		*P* Value	FDR Adjusted *P* Value	Taxonomy
6 Month Samples for 6 Month Outcome								
f__[Odoribacteraceae]	12 (13.31)	6 (75.76)	**2.67**	1.93	3.40	**<0.0001**	**0.0001**	p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__[Odoribacteraceae]
f [Barnesiellaceae]	10 (92.57)	8 (290.14)	**2.04**	1.28	2.79	**0.0001**	**0.001**	p Bacteroidetes; c Bacteroidia; o Bacteroidales; f [Barnesiellaceae]
f [Mogibacteriaceae]	15 (80.21)	6 (120.47)	**1.77**	1.04	2.50	**0.0001**	**0.001**	p Firmicutes; c Clostridia; o Clostridiales; f [Mogibacteriaceae]
f__Peptostreptococcaceae	22 (584.2)	9 (50.18)	**-2.08**	-3.45	-0.71	**0.004**	**0.036**	p__Firmicutes; c__Clostridia; o__Clostridiales; f__Peptostreptococcaceae
f__Gemellaceae	13 (74.13)	11 (23.62)	**-0.94**	-1.68	-0.19	**0.017**	0.114	p__Firmicutes; c__Bacilli; o__Gemellales; f__Gemellaceae
f Carnobacteriaceae	15 (85.06)	10 (36.09)	**-0.92**	-1.69	-0.15	**0.022**	0.122	p Firmicutes; c Bacilli; o Lactobacillales; f Carnobacteriaceae
f__Eubacteriaceae	9 (130.57)	6 (26.19)	**-0.74**	-1.38	-0.10	**0.026**	0.125	p Firmicutes; c Clostridia; o Clostridiales; f Eubacteriaceae
f__Alcaligenaceae	18 (1013.31)	11 (4759.93)	**1.80**	0.05	3.56	**0.045**	0.184	p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Alcaligenaceae

Table 4.4 Log Fold Change of Significant Taxa at Genus level for Patients with Disease Recurrence

Zero-inflated Gaussian modelling on cumulative sum scaled and normalised data at OTU level, further aggregated at family level. Only taxa that met $P \leq 0.05$ are reported.

4.3.4 Cluster Analysis

We then used hierarchical clustering to assign each sample to one of six distinct groups (see methods). This methodology allows all samples to be taken into account, reducing the dimensionality of the data. It allows visualisation of the differences in the structure of the total microbial community, which may be associated with a disease state, rather than reducing each phenotypic comparison to a single taxonomic change.

Samples within each group were enriched for bacterial families including (from left to right, Figure 4.3) the *Lachnospiraceae*, *Bacteroidaceace*, *Veillonellaceae*, *Enterobacteriaceae*, *Prevotellaceae* and a mixed profile. Two groups were associated with endoscopic outcomes at 18 months. The *Lachnospiraceae* enriched cluster and the *Enterobacteriaceae* enriched cluster were significant, with the *Lachnospiraceae* cluster associated with a protective effect (Table 4.5; Adjusted odds ratio 0.47, 95% CI 0.27-0.82; *P* = 0.007) and the *Enterobacteriaceae cluster* associated with an increased risk of recurrence (Adjusted odds ratio 6.35, 95% CI 1.24 – 32.44; *P* = 0.026).

Following identification of the two clusters of interest, we addressed if a difference in OTU diversity within the families *Lachnospiraceae* (lower OTU diversity in other clusters compared to *Lachnospiraceae* cluster) and *Enterobacteriaceae* (increased OTU diversity in the *Enterobacteriaceae* cluster compared to the other clusters) may account for difference in the risk of disease recurrence (Figure 4.4). Patients in both these groups had much higher diversity (as measured by number of OTUs within the family of interest), compared to all other patients combined (*P* <0.0001 for n OTUs in both the *Lachnospiraceae* cluster and the *Enterobacteriaceae* cluster compared to all other groups). As the abundance of *Faecalibacterium prausnitzii* has been reported influence post-operative recurrence, we specifically addressed diversity of OTUs within this genus in all clusters (Figure 4.5). [10-12] There was a significant increase in the number of OTUs mapped to the *Faecalibacterium* genus (from the family *Clostridiaceae*) in the *Lachnospiraceae* cluster compared to other clusters (*P* = <0.001).

At baseline, the cluster group assignments were tested for association with patient phenotype. The mixed profile cluster was associated with the penetrating disease phenotype (Montreal B3) at baseline (baseline OR 15; 95% CI 1.81-671.98, *P* = 0.002). The *Lachnospiraceae* cluster was associated with a lower incidence of previous

surgery (baseline; no prior surgery versus one or more previous operations; OR 0.23, 95% CI 0.06-0.84, *P* = 0.022). No cluster group was associated with smoking status.

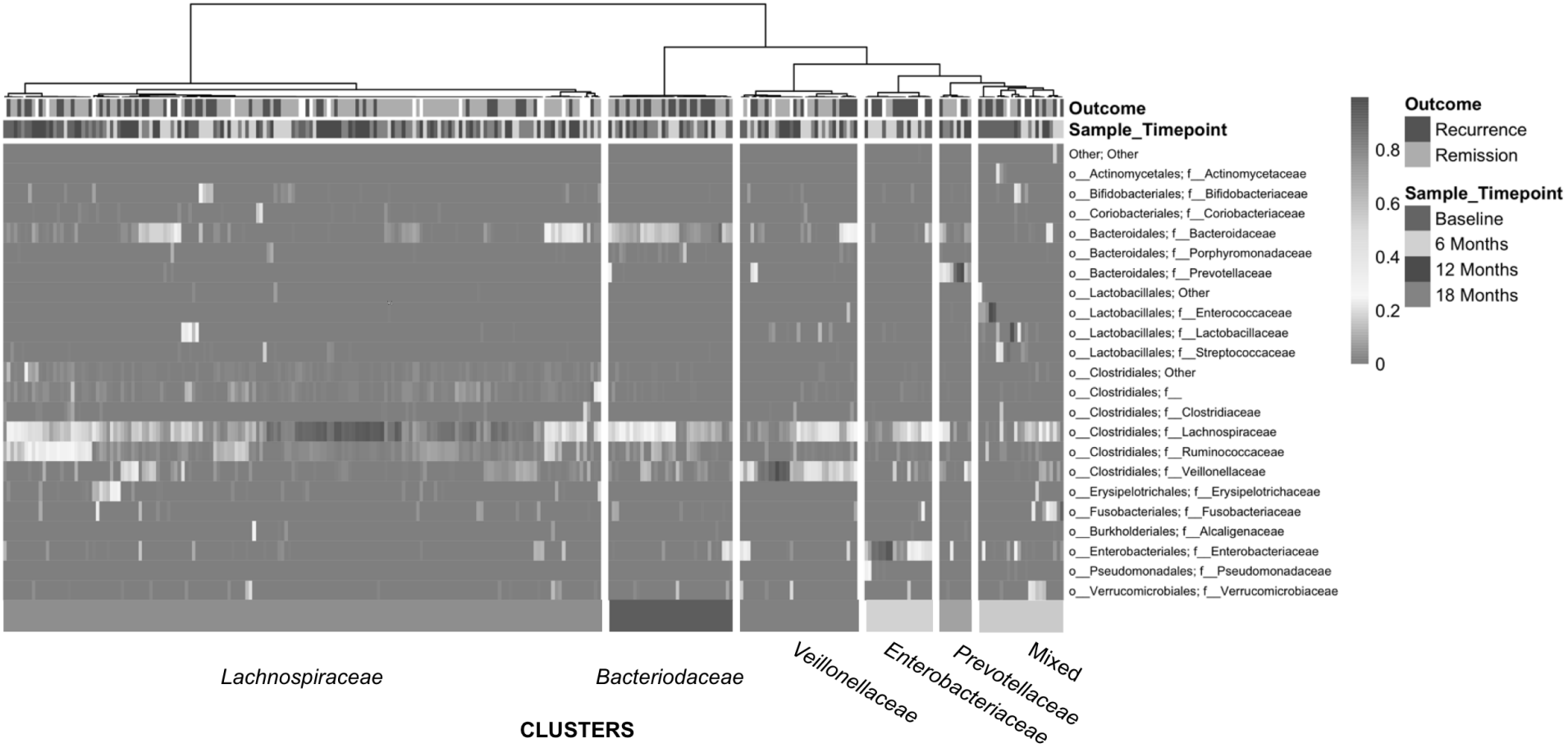

Figure 4.3 Hierarchical cluster analysis showing samples partitioning into 6 major groups based on relative abundance at family level

Annotations below the dendrogram are outcome (Recurrence ≥ Rutgeerts i2, Remission ≤ Rutgeerts i1) and sample timepoint.

Clusters	1	2	3	4	5	6
	High *Lachnospiraceae*	High *Bacteroidaceae*	High *Veillonellaceae*	High *Enterobacteriaceae*	High *Prevotellaceae*	Mixed
For 18 month outcomes by sample - adjusted for age, gender, time, baseline BMI, antibiotics and smoking						
n samples per group	148	35	28	15	8	20
n remission at 18M	99	18	16	2	5	8
n recurrence at 18M	49	17	12	13	3	12
Unadjusted OR (95% CI)	0.45 (0.27-0.75)	1.3 (0.66-2.78)	1.07 (0.48-2.37)	8.70 (1.82-41.7)	0.92 (0.21-4.0)	2.05 (0.80-5.26)
P Value	0.003	0.414	0.873	0.007	0.920	0.136
Adjusted OR (95% CI)	0.47 (0.27-0.82)	1.69 (0.77-3.73)	0.83 (0.35-1.98)	6.35 (1.24-32.44)	0.76 (0.16-3.70)	1.90 (0.69-5.26)
Adjusted P value	0.007	0.190	0.677	0.026	0.731	0.220

Table 4.5 Odds Ratios for endoscopic recurrence (Recurrence ≥ Rutgeerts i2, Remission ≤ Rutgeerts i1) at 18 months based on hierarchical clustering groups at family level using Generalised Estimating Equations

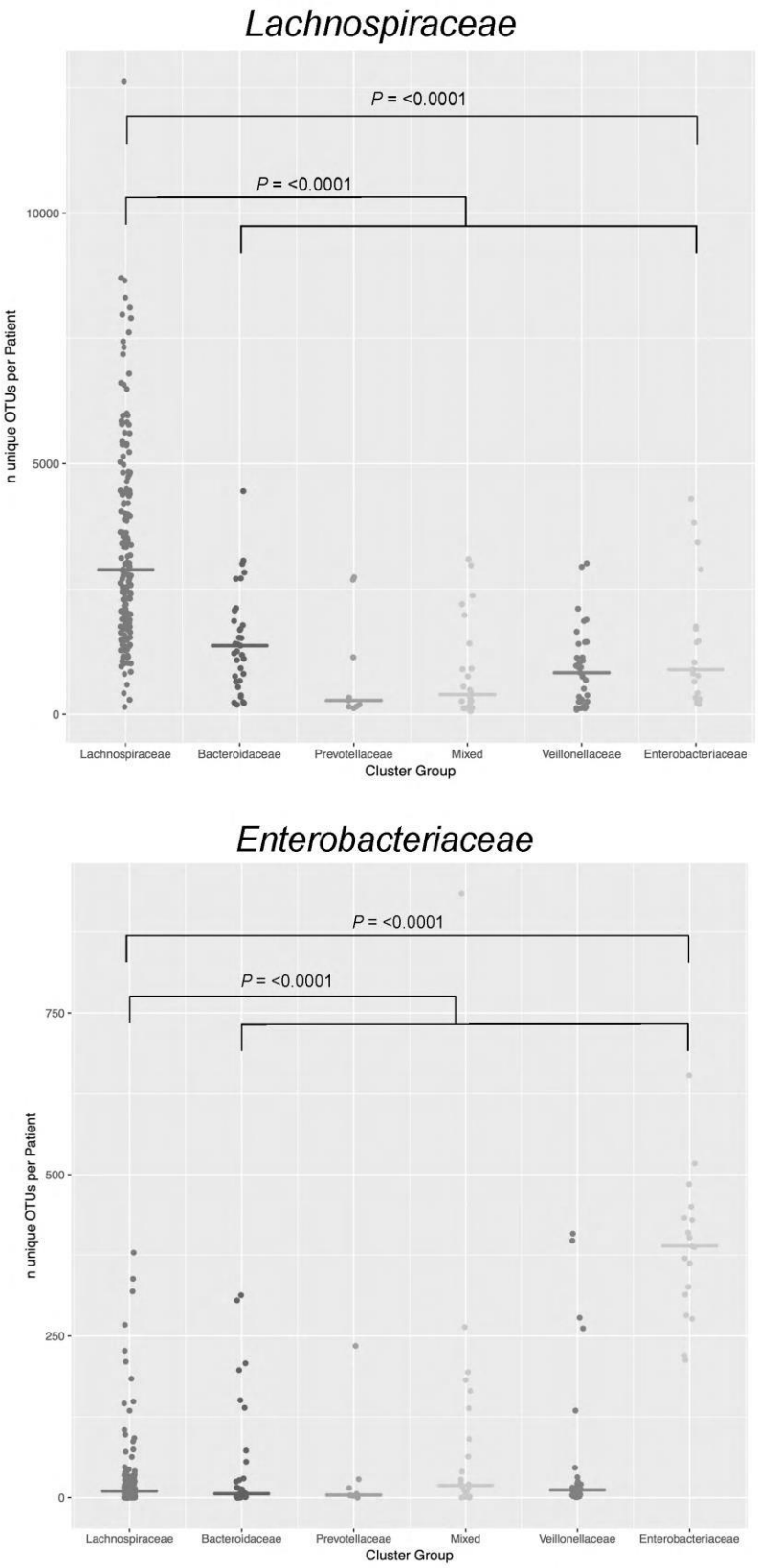

Figure 4.4 Number of OTUs within each cluster for *Lachnospiraceae* (Top) and *Enterobacteriaceae* at family level (Bottom)

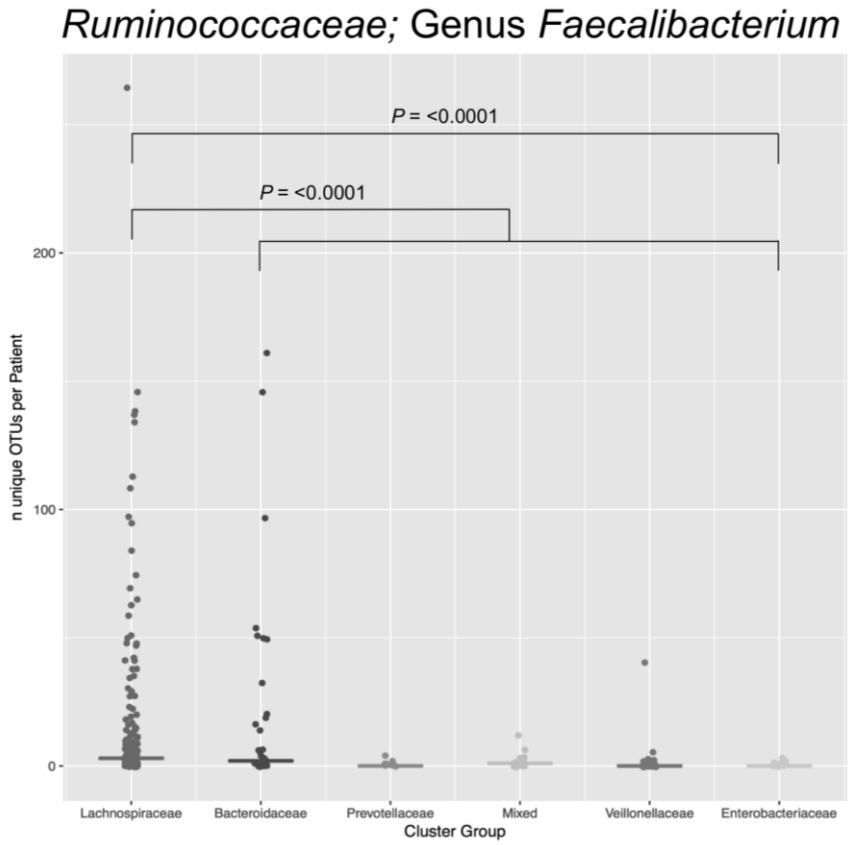

Figure 4.5 Number of OTUs within each cluster for *Faecalibacterium* at genus level

4.3.4.1 Sub-clustering of Significant Clusters

Sub-clustering at OTU level for the family level clusters predominated by *Lachnospiraceae*, *Bacteroidaceae* and *Enterobacteriaceae* was undertaken to address the contributions of individual OTU's (Figures 4.6, 4.7, 4.8). The average silhouette values for the three sub clusters were 0.53, 0.75 and 0.86 respectively. The *Lachnospiraceae* family level cluster further sub-clustered into three groups, none of which were predominated by a particular OTU. Two of the three groups were statistically significant for disease recurrence at 18 months (Table 4.6). The *Bacteroidaceae* family level cluster split into seven groups (each dominated by a different OTU, Figure 4.8).

The largest group (group 1) was associated with recurrence at 18 months, and the Greengenes OTU58029 (accession GQ448384.1) dominated. The full length representative sequence was assessed using the National Center for Biotechnology Information [NCBI] BLAST reference database. The closest match was *Bacteroides*

vulgatus strain JCM 5826 (query cover 100%, E Value 0.0, identity 95%). Sub-clustering of the *Enterobacteriaceae* group generated three clusters, with the largest cluster (cluster one) likely associated (*P* = 0.055) with recurrence and predominated by a single OTU (Table 4.6, Figure 4.7). Greengenes OTU1109844 (accession HQ883948.1) most closely matched to *Escherichia fergusonii* strain ATCC 35469 (query cover 99%, E Value 0.0, identity 96%)

Sub-Clusters	**Adj. Odds Ratio for Recurrence**	**95% CI**		***P* Value**
Lachnospiraceae Sub-cluster 1	**0.366**	0.194	0.693	**0.002**
Lachnospiraceae Sub-cluster 2	0.562	0.099	3.201	0.516
Lachnospiraceae Sub-cluster 3	**4.855**	1.195	19.726	**0.027**
Enterobacteriaceae Sub-cluster 1	**10.915**	0.952	125.163	***0.055***
Enterobacteriaceae Sub-cluster 3	1.072	0.122	9.453	0.950
Bacteroidaceae Sub-cluster 1	**4.322**	1.361	13.718	**0.013**
Bacteroidaceae Sub-cluster 2	1.777	0.012	252.947	0.820
Bacteroidaceae Sub-cluster 3	1.224	0.185	8.103	0.834
Bacteroidaceae Sub-cluster 7	1.106	0.109	11.234	0.932

Table 4.6 Adjusted Odds ratios for Disease Recurrence at 18 months for sub-cluster groups using Generalised Estimating Equations.

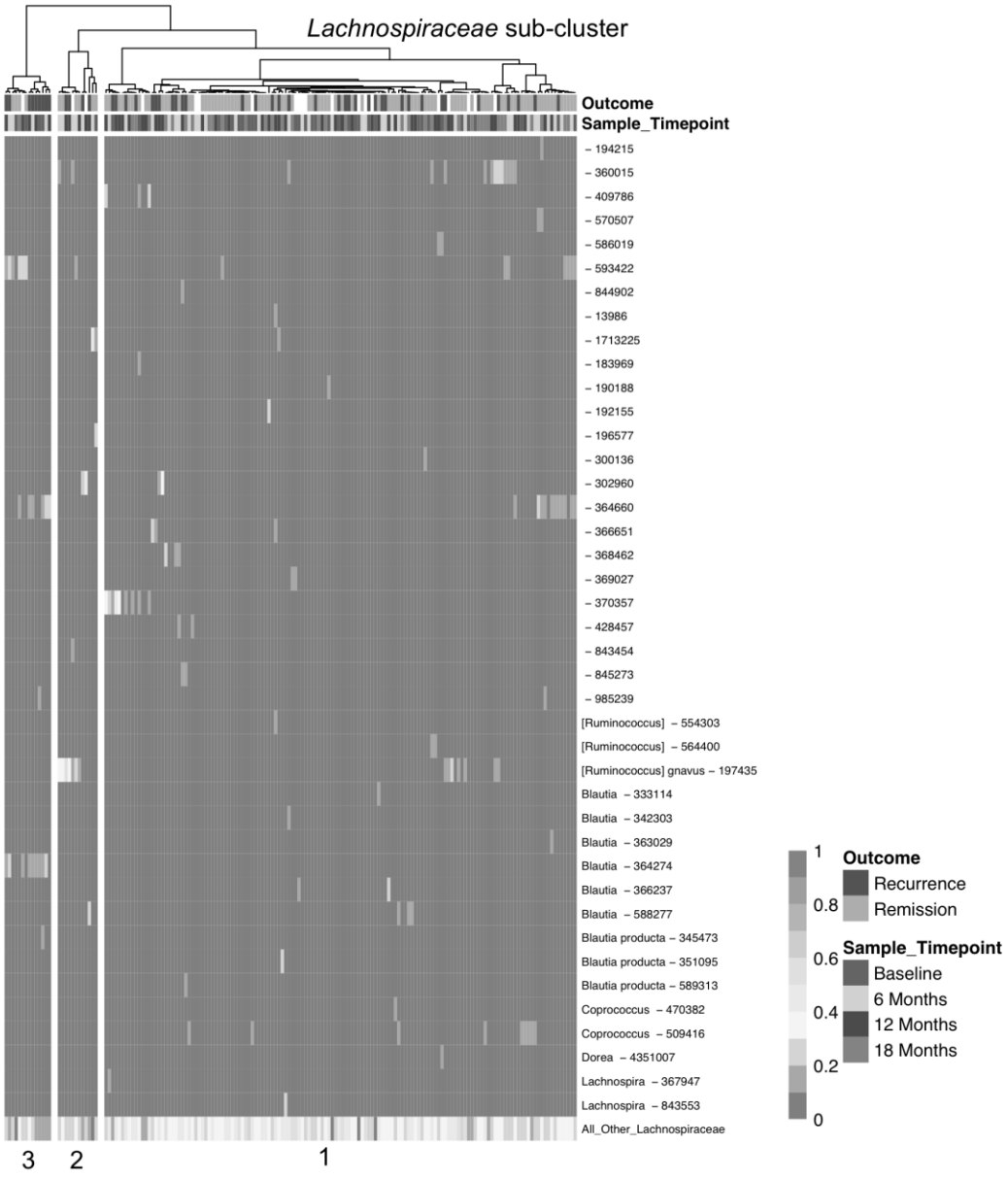

Figure 4.6 Further Hierarchical cluster analysis showing sub-clustering of the *Lachnospiraceae* family level cluster (Figure 4.3) at OTU level. OTU's that did not meet the threshold (relative abundance of at least 0.1% average, 10% abundance in at least one sample) were collapsed together into the bottom row of the heatmap. Taxonomy is given where known, or else the OTU ID number is used. Numbers refer to the sub-clusters as referred to in the text. Annotations below the dendrogram are outcome (Recurrence ≥ Rutgeerts i2, Remission ≤ Rutgeerts i1) and sample timepoint.

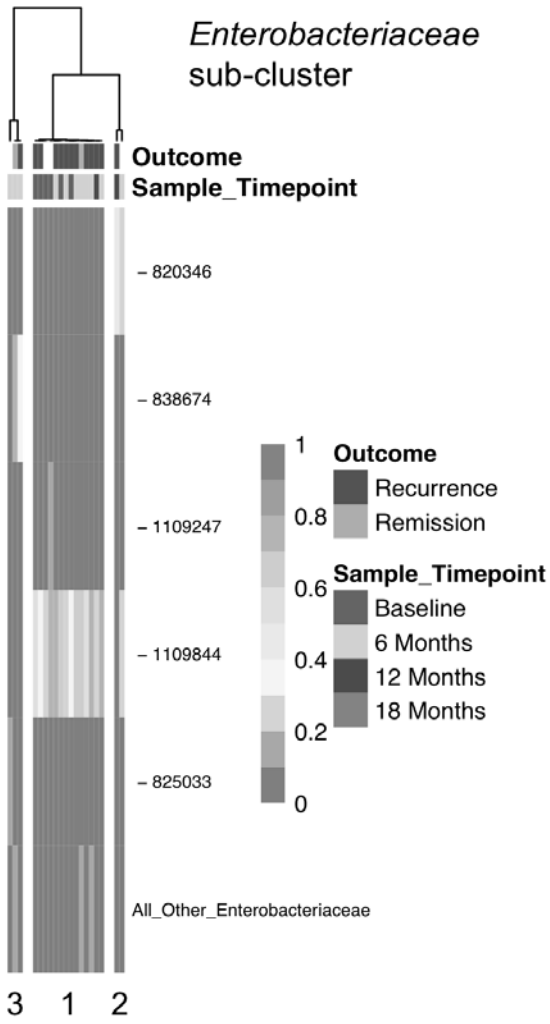

Figure 4.7 Further Hierarchical cluster analysis showing sub-clustering of the *Enterobacteriaceae* family level cluster (Figure 4.3) at OTU level. OTU's that did not meet the threshold (relative abundance of at least 0.1% average, 10% abundance in at least one sample) were collapsed together into the bottom row of the heatmap. Taxonomy is given where known, or else the OTU ID number is used. Numbers refer to the sub-clusters as referred to in the text. Annotations below the dendrogram are outcome (Recurrence ≥ Rutgeerts i2, Remission ≤ Rutgeerts i1) and sample timepoint.

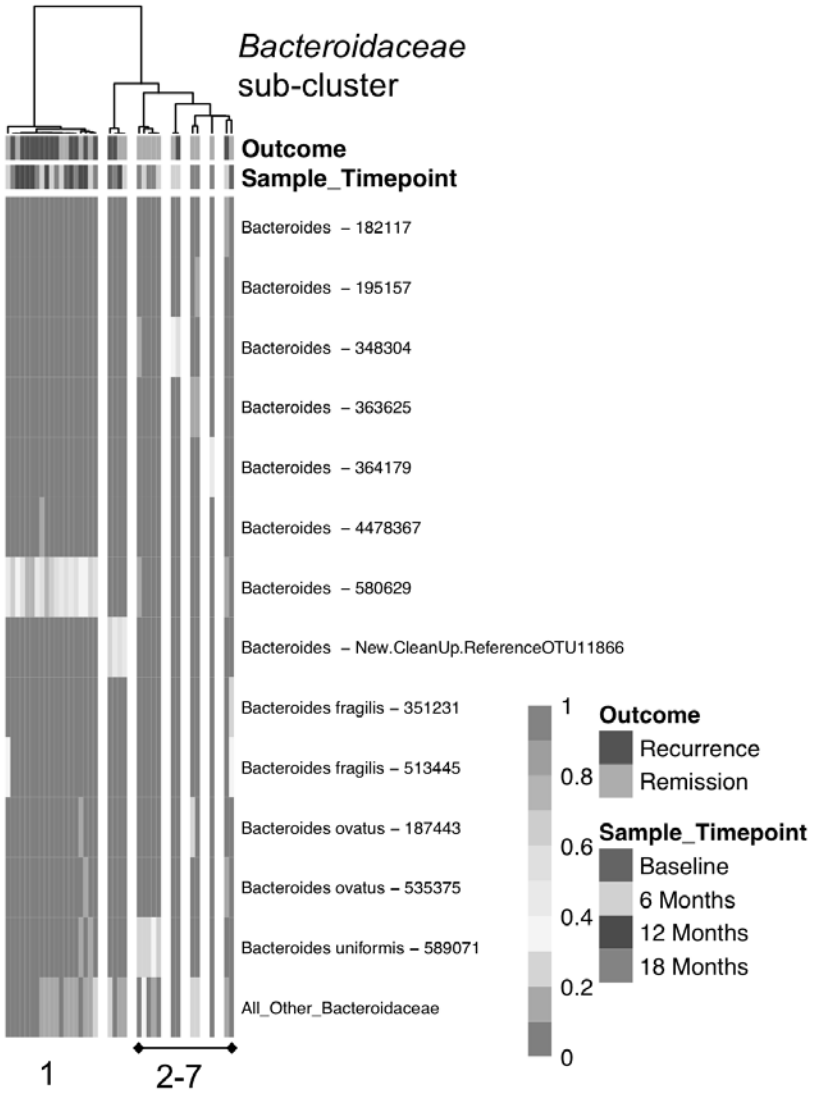

Figure 4.8 Further Hierarchical cluster analysis showing sub-clustering of the *Bacteroidaceae* family level cluster (Figure 4.3) at OTU level. OTU's that did not meet the threshold (relative abundance of at least 0.1% average, 10% abundance in at least one sample) were collapsed together into the bottom row of the heatmap. Taxonomy is given where known, or else the OTU ID number is used. Numbers refer to the sub-clusters as referred to in the text. Annotations below the dendrogram are outcome (Recurrence ≥ Rutgeerts i2, Remission ≤ Rutgeerts i1) and sample timepoint.

4.3.5 Temporal Dynamics

We addressed the temporal dynamics of the cluster groups, both at family level and within the sub-clusters combined (Figure 4.9). The *Enterobacteriaceae* cluster contained more baseline and six-month samples than 12 and 18 month samples (P = 0.0005). The mixed cluster group was predominated by baseline samples (P = 0.004), likely as a result of disease and inflammation related de-structuring of the microbiome prior to surgery.

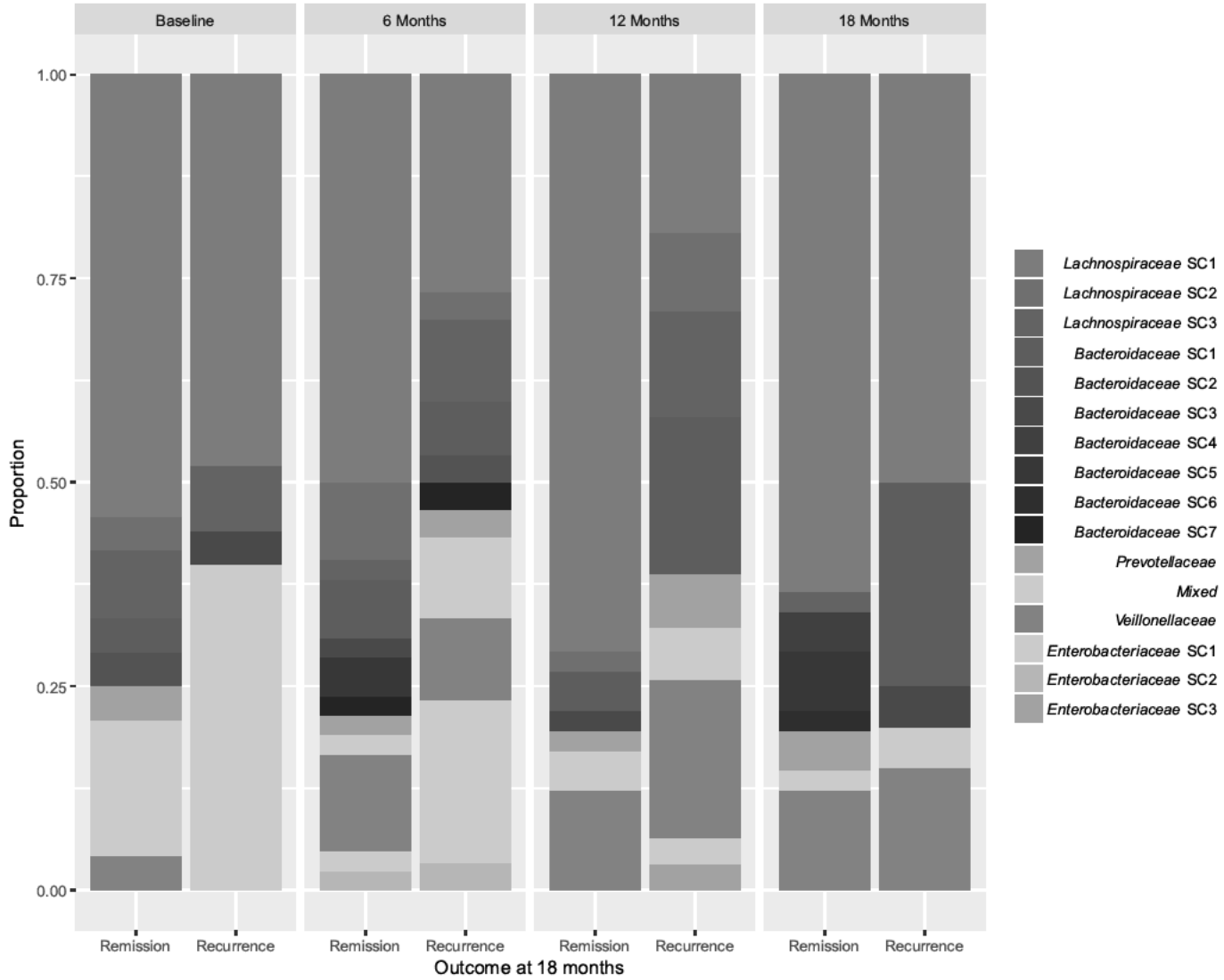

Figure 4.9 Temporal Dynamics of the Family level Clusters and OTU level sub-clusters (SC; with clusters numbered as per Figure 4.6 , Figure and Figure 4.8)

4.4 Discussion

Luminal surgery is known to alter the gut microbiome in both healthy and Crohn's Disease patients. These changes occur as a result of structural changes to the continuity of the gut, environmental modifications, pre-existing and post-operative inflammation and antibiotics administered in the immediate postoperative period. The act of performing surgery drastically alters the local environment of the resected bowel, via oxygen exposure (in open surgery) and possible alterations in peritoneal pH as a result of CO_2 insufflation (for laparoscopic surgery). However, large scale, longitudinal surveys of the post-operative faecal microbiome have not been undertaken. Here, we demonstrate that while individual taxonomic changes in specific genera may not explain post-operative disease recurrence, there are overall patterns of both resilience and recurrence that may shed light on the aetiology of recurrent disease.

Our result are consistent with trends seen by Dey *et al* and others, in that reduced abundance of genera within the *Lachnospiraceae* family at the time of surgery and subsequently, was associated with disease recurrence.[169, 218, 219] Similar work using faecal samples by Halfvarson *et al* has also demonstrated that the abundance of *Lachnospiraceae* is reduced in Crohn's patients who have undergone a resection versus those that have not had surgery, as well as compared to healthy controls.[171]

Furthermore, abundance of *Enterobacteriaceae* at the earlier timepoints (baseline and six months post-operatively was associated with an increased risk of disease recurrence, even when adjusted for patient risk factors. Enrichment for this family has been associated with active CD[220], and genera within this family (*Proteus* and *Escherichia* species) have been linked with post-operative disease recurrence in other studies looking at the mucosal microbiome.[153, 154, 168, 171] There was also a decrease in the OTU diversity of the *Faecalibacterium* genus in the *Enterobacteriaceae* cluster associated with disease recurrence, which is consistent with the observations made by Lopez-Stiles *et al* that diversity within the *Faecalibacterium* genus was reduced in Crohn's Disease patients.[221] The sub-cluster analysis highlighted two OTU's that were potentially associated with disease recurrence, an unidentified *Bacteroides* and an unidentified member of the *Enterobacteriaceae* family. Interestingly, the baseline samples were notable for the low proportion of *Bacteroidetes* seen at phylum level (6.7%), this may be as a result of the inflammatory burden pre-operatively in these patients. This baseline characteristic was seen in a small subset of these patients analysed previously, both when compared to healthy controls[222] and in patients who

recurred[170]. This depletion of *Bacteroidetes* has also been identified in other studies of Crohn's patients, and represents a marker of established disease.[104, 105, 223]

The faecal stream is far more heterogeneous than the mucosal surface, representing the sub-total of the gut microbiome. At the higher taxonomic levels, it is less likely to truly reflect a localised mucosal disease process (as it represents the total bacterial community of the gut), and this may account for the lack of specific associations at genus and species level. While the mucosal microbiome may better reflect the initiating process in CD, identification of characteristic patterns of ecological modification post-operatively further elucidates the environmental factors that may influence the development of recurrent Crohn's Disease after surgery.

The ecology of butyrate producing genera is altered by surgery; obligate anaerobes are depleted in the immediate post-operative period by environmental changes (O_2, pH, inflammation) combined with the effect of antibiotics.[148] The *Lachnospiraceae* family reside within the *Clostridium* cluster *XIVa*, and contain many keystone butyrate producing genera (such as *Blautia*, *Butyrivibrio*, *Anaerostipes*, *Dorea*, *Roseburia*, *Coprococcus* and *Eubacterium*).[224] Depletion of these obligate anaerobes alters the availability and downstream metabolism of microbiota-derived butyrate by colonocytes. The switch by colonocytes from butyrate oxidisation (when the butyrate is depleted) to fermentation of glucose results in increased luminal oxygen availability, which in turn, allows the facultatively anaerobic *Enterobacteriaceae* to expand.[148, 178] There is evidence for post-surgical 'blooms" of the *Enterobacteriaceae* family, even in patients without inflammatory bowel disease. A study addressing microbial alterations in patients with an ileostomy (following small bowel transplant) demonstrated significant expansion in the *Enterobacteriaceae* and *Lactobacillaceae*, as a result of increased oxygen favouring the facultative anaerobes, and reducing populations of obligate anaerobes.[134] Antibiotics (as prescribed in these patients) also influence microbial ecology via respiratory pathways and depletion of keystone bacterial families, and some antibiotics have been linked with enterobacterial expansion.[176, 225]

In addition to alterations in oxygen availability, there are also increases in the availability of terminal electron acceptors (and donors) for microbial facultative anaerobic metabolism by the *Enterobacteriaceae*, such as Nitrate, Nitrite, S-oxides (tetrathionate), N-oxides and fumarate.[173, 179, 180] This occurs as a result of host inflammatory responses, and is postulated to be a consequence of inflammation rather than a cause.[173, 226] Recruitment of neutrophils, and subsequent respiratory burst in the

inflamed bowel releases reactive oxygen species (ROS) that, with endogenous sulphur containing compounds, react to produce tetrathionate, an electron acceptor for *Salmonella* and *Proteus* spp.[116, 180] Breakdown of colonocytes provides further alternative respiratory electron acceptors via ethanolamine obtained from phospholipid/cell-wall breakdown[181], as well as trimethylamine derived from phosphotidylcholine.[182, 183] Thus, the proportion of samples within the *Lachnospiraceae* cluster increases in the later timepoints (12 and 18 months) may indicate that the ecologic pressures of the perioperative period (inflammation, oxygen availability and antibiotic exposure) have somewhat resolved in patients maintaining remission.

Two different analytic approaches were undertaken in this study, taxonomic analysis – using differential abundance of normalised count data between outcomes, and hierarchical clustering on relative abundance, identification of cluster groups and association testing using generalised estimating equations. These approached produced contrasting results, likely as a result of the different methodologies. Taxonomic analysis seeks to define the increase or decrease in the abundance of many individual taxa, whereas the hierarchical clustering allows comparisons of community structure, particularly where a particular bacterial family dominates samples. The differences seen between the analyses are likely accounted for by a threshold effect, where a predominating bacterial family is more important than individual taxa.

Limitations of this study include the heterogeneity of the drug treatment provided in the post-operative period, limiting our ability to correct for therapy. Nearly 80% of patients completed a full course of metronidazole post-operatively, and although this is standard prophylaxis after surgery for CD it may confound some comparisons.

Further investigation of the functional capacity of the post-operative microbiome may elucidate the differences in microbial respiratory gene function in patients with recurrence. This may highlight the post-operative utilisation of alternative respiratory pathways available to pathobionts in response to environmental factors, providing possible targets for prevention of post-operative recurrence. Greater sample sizes are required to address the taxonomic shifts at genus and species level, with more intensive sampling to truly reveal the fine temporal shifts that occur after surgery.

In conclusion, we have identified broad microbial profiles at family level that are associated with remission after surgery for Crohn's Disease (enrichment of *Lachnospiraceae*) and with disease recurrence (enrichment for *Enterobacteriaceae*). These complementary findings may represent two characteristic and intrinsically linked patterns associated with the aetiology of Crohn's Disease recurrence. Further work to characterise the functional characteristics of the microbial alterations in both the mucosa and the faecal stream is warranted

5 *Proteus* as Putative Gastrointestinal Pathogens - A Systematic Review

5.1 Introduction

Proteus species are members of the *Enterobacteriaceae* family of bacteria. Most commonly they are recognised clinically as a cause of urinary tract infections. Although *Proteus* is likely commensal in the gastrointestinal tract, their abundance as a proportion of the microbial community is very small (<0.05%).[123] As a result, their detection in disease states using 16S profiling, and possibly metagenomics, may have rendered *Proteus* spp. undetectable due to bioinformatic abundance thresholds.

The recent identification of *Proteus* spp. as a potential pathogen in Crohn's disease recurrence after intestinal resection[153, 154] serves as a stimulus to examine its potential role as a gut pathogen. This review aims to provide an overview of the genus *Proteus* in terms of its known virulence factors, as well as to collate the evidence surrounding the role of *Proteus* spp. in the pathophysiology of gastrointestinal diseases

5.2 Search Methodology

An electronic search of the English language medical literature was conducted using Medline (Ovid) and PubMed to identify relevant literature published up to January 2017. The search strategy used a combination of the following pre-specified MeSH headings and keywords alone or in combination: "*Proteus*", "gastrointestinal", "gastrointestinal tract" and "digestive". Boolean operators ("not," "and," "or") were used in succession to narrow or widen the search. Additional searches were performed within the MeSH headings for the more specific terms *Proteus* or *Proteus penneri* or *Proteus mirabilis* or *Proteus vulgaris* or *Proteus* infection. Searching abstracts from relevant international conferences over the last 5 years was undertaken to obtain contributions not identifiable through electronic searching. In addition, reference lists of reviews and original research articles were assessed.

Inclusion criteria included clinical and laboratory (mechanistic) papers referring to *Proteus* spp. in relation to gastrointestinal disease. Articles assessing *Proteus* spp. in relation to renal or bladder infections, or animal models, were only included when there was direct relevance to gastrointestinal disease pathogenesis.

Exclusion criteria included: non-English language or publications not available as full text, non-gastrointestinal studies, studies utilising culture of *Proteus* species to assess antibiotic activity, studies not investigating *Proteus* bacteria, studies in animals not relevant to humans, and publications relating to bacteria previously listed as *Proteus* that now are recognized as another bacterial genus (e.g. *Proteus morganii*, now *Morganella morganii)*.

5.3 Search Results

721 individual abstracts were screened for inclusion, with 306 meeting inclusion criteria (Figure 5.1). A further 109 papers were excluded after full text consideration, leaving 197 papers included in this review. Within the reviewed abstracts, 26 relevant papers were identified outside of the database search, from reference lists or conference abstracts. Of these 26 papers, eight were previously identified outside of the search criteria. These large microbiome surveys reported only briefly on *Proteus* species, such that these references would not be indexed by the databases on the basis of related key words.

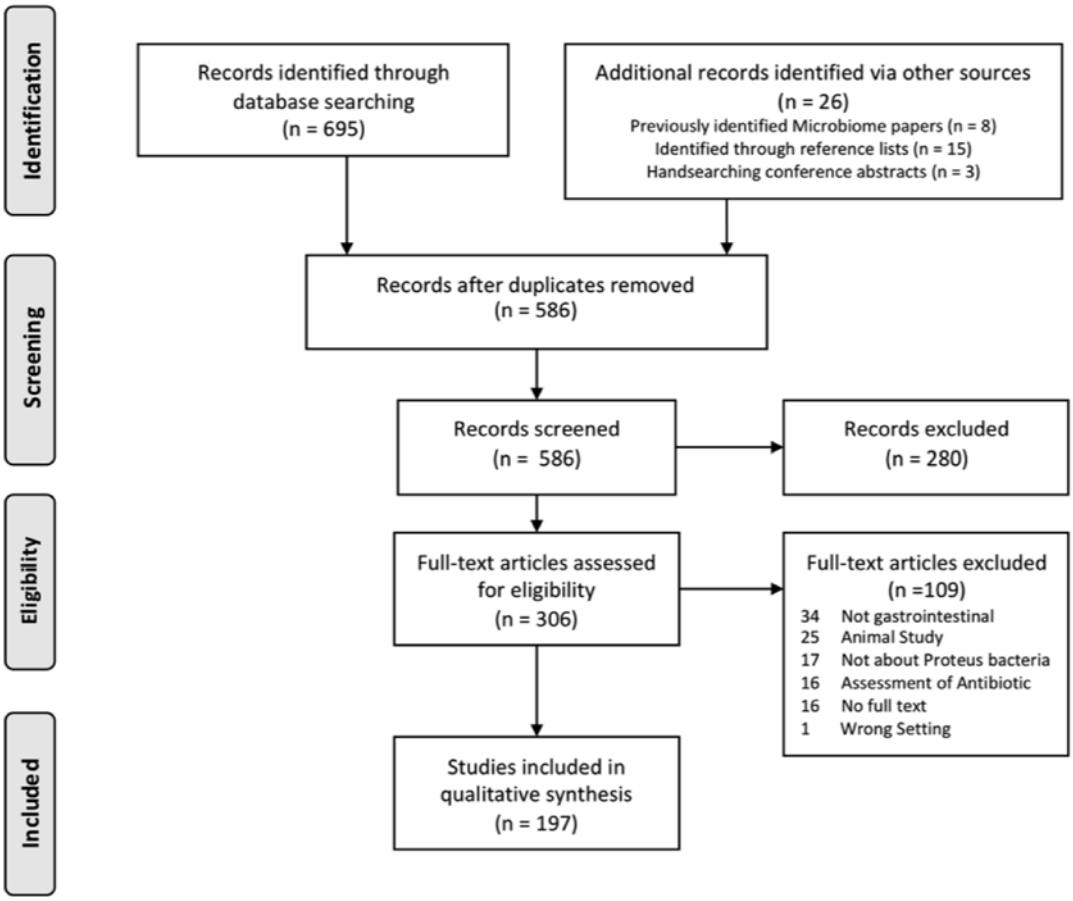

Figure 5.1 Literature search - PRISMA Flow Chart

5.4 Characteristics of the *Proteus* Genus

Proteus spp. are gram-negative bacteria belonging the *Enterobacteriaceae* family, are common commensal gastrointestinal microbiota.[227] The first isolates were reported and characterised by Hauser in the late 19th Century, and are readily cultivated in the laboratory.[191] The genus is currently comprised of P. mirabilis, P. vulgaris, *P. penneri, P. hauseri, P. terrae, P. cibarius* and *P. myxofaciens*, along with the unnamed genomospecies 4, 5, and 6.[189, 228-231] In humans, all current members of the genus except for *P. cibarius, P. terrae*, and *P. myxofaciens* have been isolated from clinical specimens.[191, 227, 228, 230, 232] Typically, the human gut is colonised by various combinations of P. vulgaris, P. mirabilis, and P. penneri, but they comprise less than 0.05% of the gut microbiota of healthy subjects (Figure 5.2). [123, 233]. While *Proteus* spp. are widely recognised as pathobionts and the gut is the reservoir of these bacteria, the research focus on the genus has been their role in urinary tract infections rather than intestinal manifestations.[234, 235]

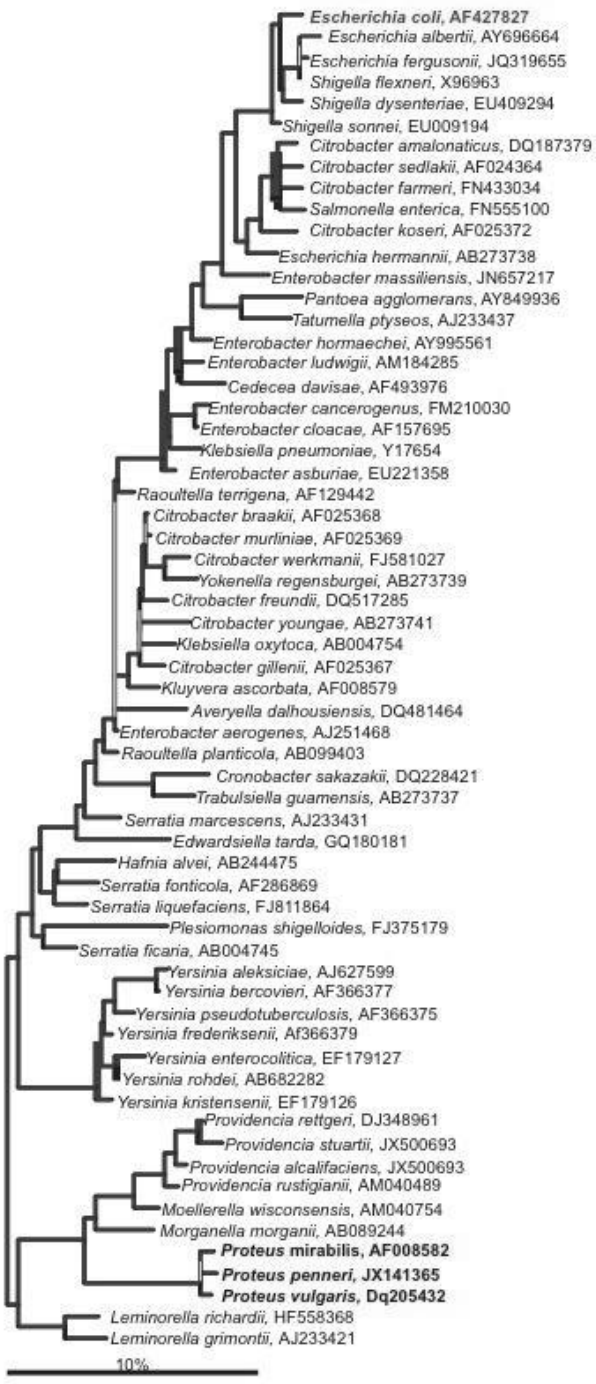

Figure 5.2 Phylogenetic Tree showing the species that colonise the human gastrointestinal tract from the *Enterobacteriaceae* family GenBank Accession Numbers of the16S rRNA gene sequence are provided for each species and the family names are indicated. *E. coli* is highlighted in green, with the *Proteus* genus shown in blue. Modified with permission from [224]

Many recent studies of the gut "microbiome" in health and disease have revealed that there are gross alterations in the relative proportions of key bacterial taxa associated with active disease, which is generically referred to as "dysbiosis". With specific reference to inflammatory bowel diseases (IBD) one of the hallmarks of "dysbiosis" is the expansion of the representation of the phylum *Proteobacteria*,[236] and members of the family *Enterobacteriaceae* such as *Escherichia*, *Shigella*, *Salmonella* and *Klebsiella* spp. have received due attention. The possible contribution of *Proteus* spp. to intestinal disease and infections has been somewhat neglected. Research into the virulence of *Proteus* spp. in the urinary tract using the bacteriology of ileal conduits[237] and intestinal segments[238, 239] for bladder augmentation, suggests that *Proteus* spp. should be more closely examined for their potential as gastrointestinal pathogens.

5.5 *Proteus* Pathogenic Features

Proteus species are short (1.5 – 2 μm) straight rods that demonstrate dimorphism as "swimming" and "swarming" forms, as do some other members of the *Enterobacteriaceae*.[190] Swimmer cells predominate in liquid environments, as single cells with 4-10 peritrichous flagella (Figure 5.3, bottom right).[191, 235] However, when these cells are either placed in a viscous environment or on a solid surface, they undergo differentiation to the filamentous, multinucleated, highly flagellated swarmer cell (Figure 5.3, top and bottom left). *Proteus mirabilis* can undergo swarming differentiation at much higher concentrations of agar (1.5-2%) than other swarming bacteria.[240] When *Proteus* spp. swarm there is also a dramatic increase the production of secreted proteins including virulence factors such as the protease ZapA.[190, 198, 241] The swarming phenotype can also be induced by anaerobicity[242], and amino acids, in particular glutamine. [243, 244] Fujihara *et al* (2011) also reported that a more acidic pH, as might be expected to occur in the proximal small bowel and caecum, dramatically increased swarming behaviour.[130, 245, 246] Swarming has been shown to be an important factor in intracellular invasion and persistence, with 15-20 fold more swarmer cells than swimmer cells capable of intracellular invasion of uro-epithelial cells.[247]

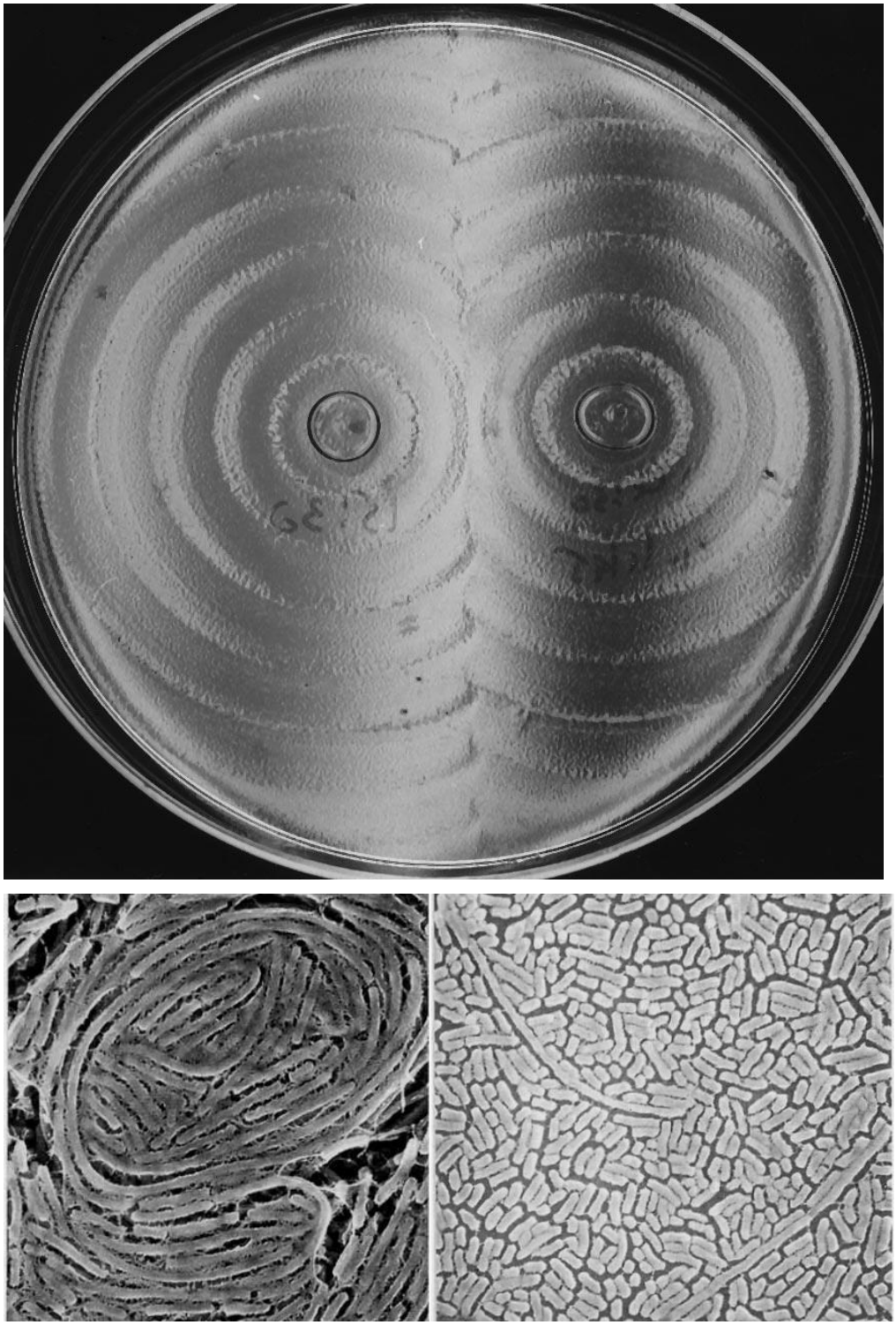

Figure 5.3 Visual morphology of *Proteus mirabilis*

Top: A strain of *P. mirabilis* inoculated twice one hour apart, demonstrating the macroscopic characteristic bulls-eye pattern produced by periodic swarming. With permission from [248]. Left. Interacting *P. mirabilis* swarmer cells. Right. A combination of swimmer and swarmer cells within a biofilm. With permission from [194]

5.5.1 Adhesion and Mucosal Attachment

Adhesion to epithelial surfaces is essential to the pathogenesis of *Proteus* infections, both in the urinary and gastrointestinal tract. Sequencing of the *Proteus mirabilis* strain HI4320 revealed 17 fimbrial gene sets (operons), more than any other bacterial genome currently characterised.[196, 249, 250] *Proteus mirabilis* can produce at least six different types of fimbrae (Table 5.1), including Mannose- resistant, *Proteus*-like (MR/P fimbrae), mannose-resistant *Klebsiella*-like fimbriae (MR/K), nonagglutinating fimbriae (NAF, also known as Uroepithelial cell adhesin (UCA)), ambient temperature fimbrae (ATF), *P. mirabilis* P-like pili (PMP) and P. mirabilis fimbriae (PMF).[191, 250] These fimbrae and adhesins play a major role in the formation of bacterial biofilm, a common complication of both urinary and gastrointestinal instrumentation.[251]

Kuan *et al* (2014) compared the 17 individual chaperone-usher fimbrial operons across the seven sequenced *P. mirabilis* strains as well as 58 clinical isolates, and showed 99% conservation in 13 of 17 fimbral operons, demonstrating that these genes are highly conserved across strains from varied clinical sites.[250] Of these, it is likely that at least two, and up to six of the characterised fimbrae can be assembled on the cell surface at any one time.[250, 252] Further work is needed to determine if the other fimbral operons are expressed, and under what conditions.

Fimbral Type	Structure		Contribution to Gastrointestinal Pathogenicity	Refs.
Mannose- resistant, *Proteus*-like (MR/P)	7-8nm "thick" channelled fimbrae	Important for epithelial cell adhesion MR/P expression undergoes phase variation allowing for a molecular "switch" that turns on/off the expression of the *mrp* operon depending on environment and oxygen availability– e.g. ON in bladder colonisation, OFF in kidney colonisation. MR/P fimbrae are potent immunogens and potential vaccine candidates. MR/P^+ *Proteus* strains are more genotoxic and cytotoxic to bladder/kidney derived epithelial cells than MR/P^- mutants	Expression would allow mucosal cell adhesion in the gastrointestinal tract and contribute to intestinal persistence The repeating structure of flagellin proteins contributes to immunogenicity in the gut The MR/P^+ *Proteus* strains are also likely more cytotoxic to intestinal epithelial cells	253-255
Mannose-resistant *Klebsiella*-like fimbriae (MR/K)	4-5nm "thin" non-channelled fimbrae	Expression is more common in *P. penneri* strains than *P. mirabilis*	*P. penneri* has not been linked with gastrointestinal pathogenicity	252, 256
Uroepithelial cell adhesin/Nonagglutinating fimbriae (UCA/NAF)	4nm "thin" non-channelled fimbrae	Recognises Glycolipids including Asialo-GM1, Asialo-GM2, lactosyl ceramide and galectin-3. Not found in *P. vulgaris*	Important for epithelial cell adhesion, may be the primary adhesion for intestinal colonisation	257-259

Fimbral Type	Structure		Contribution to Gastrointestinal Pathogenicity	Refs.
Ambient temperature fimbrae (ATF)		Highly expressed at 23°C, moderately expressed at 37°C Not considered to contribute to UTI or biofilm formation, may be more important for enteric colonisation Believed to be important for survival and persistence outside of a mammalian host, at room temperature	The structural subunit AtfA has significant homology with the major subunit of type 1 fimbriae from other enteric pathogens such as *S. typhi*	[251, 257, 260-262]
P. mirabilis P-like pili (PMP)		Identified in uropathogenic *P. mirabilis* strains isolated from dogs. Not present if *P. vulgaris.* PmpA fimbral subunits show high homology to the P-fimbriae from Uropathogenic *Escherichia coli*. Cell surface assembly has not been confirmed		[263]
P. mirabilis fimbriae (PMF)		PMF are important in biofilms, and responsible for formation of higher volume biofilms		[251]

Table 5.1 Fimbrae and pilli expressed by *Proteus* species

Swarming behaviour has been shown to repress the expression of MR/P fimbrae and NAF fimbral operons (and therefore epithelial attachment), allowing up-regulation of motility. The regulation of motility and the expression of adhesion are tightly coupled; of the 17 fimbral operons, at least 10 gene clusters possess a homolog of the gene MrpJ, a repressor of motility.[264] MrpJ down regulates the flagellar master regulator flhDC via binding to the promoter sequence.[264] When these cells revert back to the swimming morphology, expression of MR/P and NAF fimbrae returns.[251, 265] Furthermore, the induction of MR/P fimbrae appears coupled to the availability of oxygen.[266] MR/P fimbrae are phase variant, and can be "switched" on or off by a site specific DNA recombinase (MrpI) that inverts a promoter region flanked by inverted repeats. This "invertible element" switches on or off the MR/P fimbral operon, depending on environmental conditions. There appears to be a growth advantage to MR/P fimbral expression in low oxygen conditions such as would be present in the intestinal tract, contributing to the adhesiveness and persistence of *Proteus* species in the gut.[266] The adaptation of *Proteus* species to mucosal surfaces by way of both fimbral expression (for adherence) and swarming motility could increase the invasiveness, persistence and pathogenicity of these species in the gut (Table 5.1).

5.5.2 Urease

The urease enzyme is a microbiological adaptation to metabolise urea, the most abundant nitrogenous waste product of human metabolism.[267] As with *Helicobacter pylori*, the presence of this enzyme confers a survival advantage through increasing the local pH of the environment, allowing urease positive organisms to survive in more acidic environments. *Proteus* spp. have an wide pH range for growth, from 5-10, with an optimal pH of between 7-8.[230] Urease activity has been confirmed in *P. mirabilis, P. vulgaris* and *P. penneri,* and production is regulated by both the environmental concentration of substrate (urea) and increased chromosomal transcription in swarming cells.[197, 268, 269] Colonisation of the upper gastrointestinal tract by urease producing *Proteus* species may cause occasional false positive results for the [13C]Urea breath test (UBT), especially if the patient has already been treated with proton-pump inhibitors and antibiotics for *H. pylori* eradication.[270] Given that urease is a known contributor to the pathogenesis of *H. pylori* in the upper gastrointestinal tract, it may contribute to the pathogenesis of *Proteus* spp. in the gut in the setting of environmental perturbations (such as changes in pH).

5.5.3 Haemolysins

The *Proteus* genus produces two distinct cytotoxic haemolysins, HpmA and HlyA.[197, 271, 272] *P. mirabilis* and most *P. vulgaris* strains produce only HpmA, most *P. penneri* strains produce HlyA and a few isolated *P. vulgaris* strains produce both HpmA and HlyA.[271, 273, 274] HpmA has been shown to lyse erythrocytes, bladder epithelial cells, B-cell lymphoma cells and monocytes, while HlyA can lyse erythrocytes, fibroblasts and neutrophils. [272, 273] HpmA is a cell-associated haemolysin, encoded on the *Hpm* locus along with HpmB (an activator and chaperone of HpmA). Expression of these haemolysin proteins is tightly coupled to the swimming-swarming cycle, with swarming cells being 18-fold more cytotoxic than swimmer cells.[247] HpmA has also been shown to lyse erythrocytes under anaerobic conditions, and at multiple temperatures.[275] The contribution of haemolysins to gastrointestinal pathogenesis would occur through lysis of innate immune cells, and induction of NLRP3 inflammasome and downstream IL-1β release (see section 1.6.7).

5.5.4 Intracellular invasion and persistence

Intracellular invasion by *Proteus mirabilis* has mainly been assessed using cellular invasion assays in cell lines, ranging from uroepithelial cells to colonic cell types. The origin of the *P. mirabilis* isolate influences the invasiveness of *Proteus* species in laboratory studies, with faecal isolates showing higher invasion efficiencies.[276]
In a cell-based urinary tract model, swarming cells were 15 fold (0.18% intracellular localisation) more invasive to uroepithelial cells than swimmer cells (0.012% entry).[247] After invasion of uroepithelial cells swarmer cells start to divide, develop septa and differentiate back to an average of 50-300 swimmer cells within the cytoplasm.[247] *P. mirabilis* has been identified as intracellularly invasive in a number of cell lines summarised in Table 5.2. There are differences in the intracellular invasion and uptake pathways depending on cell type, with *P. mirabilis* having a single membrane within urothelial cells and a double membrane within intestinal cells.[238, 276] These mechanisms contributes to effective intracellular colonisation (cytoplasmic colonies), evasion of the host immune system, and resistance to antibiotics.[238]

Cell Line	Cell type	Reference
CaCo-2†/ CaCo-2BBe†	Colorectal adenocarcinoma/ C2BBe1 (brush border expressing) Models for apical microvilli and tight-junctions	238, 239, 277, 278
HT29†	Enterocytes - Undifferentiated or differentiated (polarised, in absence of glucose)	238, 239, 277, 279
HT29-18N2†	Mucus-secreting differentiated cells	238, 239
HT29-FU†	Mucus-secreting differentiated cells	
HT29-MTX†	Mucus-secreting differentiated cells	
INT407†	Embryonic Intestinal epithelial cells (possible HeLa contaminated line)	276
HCT-8†	Ileocecal colorectal adenocarcinoma	
T24	Transitional cell bladder carcinoma	
HeLa	Cervical Carcinoma	280
Vero	African Green Monkey Kidney Cells	
L-929	Mouse fibroblasts	
Human Blood Lymphocytes		

Table 5.2 Cell culture lines capable of intracellular uptake of *Proteus mirabilis*

Gastrointestinal cell lines are shown with a †

5.5.5 Immune evasion

In addition to intracellular invasion *P. mirabilis* also secretes an extracellular metalloprotease (ZapA) that has been shown to degrade a wide range of substrates (Table 5.3).

Substrates			Location	References
Structural cell components	Actin, β-tubulin, fibronectin, collagen, laminin		All cells	198
Innate Immune components	Immunoglobulins	IgA1, IgA2, IgG		198, 281
	Complement proteins	C1q, C3		198
	Defensins	Human Beta Defensin 1	Epithelial surfaces, small and large bowel	198, 282, 283
	Cathelicidin peptide	LL-37	Epithelial surfaces, Wounds	198, 284

Table 5.3 Substrates degraded by *Proteus mirabilis* ZapA metalloprotease

This enzyme has a key role in *Proteus* species evading innate immune destruction by proteolytic digestion of secretory IgA, IgG and other cellular components in the urinary and gastrointestinal tract.[198, 241] Kerr et al (1995) showed ZapA cleavage products (IgA and IgG) in the urine of patients with P. mirabilis, reflecting protease activity in vivo.[285] The expression of this protein has been shown to be tightly correlated with the cellular differentiation from swimmer to swarming cells by *P. mirabilis*, with increased transcription of the ZapA locus.[241] ZapA hydrolyses human β-defensin 1, a constitutively expressed innate immune antimicrobial peptide that is expressed in the colonic epithelium, as well as secretory IgA.[198, 241] The expression of ZapA in the gut would provide a survival advantage to *Proteus* spp. by perturbing host immune responses and increasing the likelihood of persistent colonisation.

5.5.6 Endotoxin and Flagellins

As a gram-negative pathogen, *Proteus* species possess similar intrinsic characteristics to other *Enterobacteriaceae* such as *E. coli* and *Salmonella* Typhimurium, including production of flagellin and the pro-inflammatory cell wall component lipopolysaccharide (LPS).[286] A constituent of LPS, Endotoxin (Lipid A) is highly immune-stimulatory.[287, 288] Endotoxin is sensed by the innate immune system (specifically Toll Like Receptor 4) and activates MyD88 and downstream signalling of NF- κB.[288] This, in turn, triggers a pro-inflammatory cytokine release that can perpetuate acute sepsis.[289] In addition, bacterial flagellin, the repeating protein subunits from which flagella are built, are highly immunogenic due to their 3D structure. Bacterial flagellin is sensed by Toll Like Receptor 5, again triggering downstream MyD88 signalling and activating NF- κB.[288] These features contribute to the overall pathogenicity of *Proteus* spp. via stimulation of the host innate immune system by bacterial products.

5.5.7 Conjugation, Plasmid Acquisition and Antibiotic Resistance

Proteus species are inherently antibiotic resistant. Resistance to polymyxins is mediated via amino acid substitutions in the cell wall lipopolysaccharide that inhibit their activity.[290] They also possess intrinsic resistance to colisitin, tigecyline and tetracycline .[291] Sequencing of *P. mirabilis* strain HI4320 has demonstrated the presence of genes for a conjugal transfer pilus, which allows horizontal genetic transfer of plasmids encoding antibiotic resistance.[196]

While most species of *Proteus* remain sensitive to a range of antibiotics, a future challenge will be acquired antibiotic resistance by *Proteus* species. In 2007 – 2008, reports of *P. mirabilis* strains acquiring *Salmonella* Genomic Island-1 (SGI1) were published from groups in China and Palestine.[292] SGI1 is a mobile genomic element first identified in *Salmonella* Typhymurium that integrates into the recipient chromosome, and carries multiple genes encoding resistance to streptomycin, trimethoprim, tetracycline, sulphonamides, chloramphenicol, fluoroquinolones and a broad spectrum of β-lactam antibiotics.[292] A further SGI1 positive *P. mirabilis* strain (NKU) has also been identified in Europe.[292] Recently an SGI1 positive *P. mirabilis* strain acquired the a plasmid containing the New Delhi metallo-β-lactamase 1 gene leading to the identification of the extensively drug resistant (XDR) *P. mirabilis* strain PM58, resistant to all antibiotics used for *Enterobacteriaceae* except aztreonam.[291] A recently sequenced isolate *P. mirabilis* (NO-051/03) from a patient with a soft tissue

infection in Europe had acquired the resistance gene for trimethoprim, β-lactams, phenicols, sulphonamides and aminoglycosides.[293]

In gastrointestinal disease, the antibiotic sensitivity profile of *Proteus* species is relevant to pathogenicity in conditions that may be exacerbated by antibiotic perturbation of the gut microbiome. Given the potential for a "bloom" of *Enterobacteriaceae* (including *Proteus* species) in both the presence of an inflammatory process[226] and perturbation of the enteric environment via surgery[294], the use of antibiotics should be considered as a possible potentiating factor.

5.6 Proteus Spp. as Gastrointestinal Pathogens

5.6.1 Colonisation by *Proteus* species

Proteus species are known human digestive tract commensal organisms with abundance varying according to location. Colonisation occurs early. Infants from Sweden and Pakistan were assessed for *Enterobacteriaceae* based on mode of delivery (vaginal versus caesarean) and breastfeeding behaviour.[295] Caesarean births in Pakistan were associated with *Proteus* species colonization within 3 days, with 11 of 21 caesarean-delivered and 1 of 9 vaginally-delivered infants positive for *Proteus* spp. (P = 0.049).[295]

In healthy subjects, Zilberstein et al (2007) cultured mucosal samples from the upper (n = 20) and lower (n = 24) digestive tracts of healthy controls. *Proteus* species were present in 8% of gastric samples, 46% of duodenal and jejunal samples, 19% of ileal samples, 13% of caecal samples, 38% of samples from the transverse colon.[233]

Muller (1986) compared the recovery of *Proteus* species from the stool of 1422 healthy subjects and 1271 patients with diarrhoeal diseases. *P. mirabilis* was identified in 2.7% of healthy subjects, a likely underestimate of colonization based on the epithelial preference of *P. mirabilis*.[195] *Proteus penneri* and *P. vulgaris* were isolated from 0.9% and 4.2%, respectively, of the same population.[195]

Proteus species, especially P*. mirabilis*, are often antibiotic resistant, conferring a survival advantage when colonising the gastrointestinal tract. In a study of multiple resistant gram-negative bacteria in the rectum of long term care patients 52 drug resistant strains were identified, of which 15 were *P. mirabilis*. 87% of patients colonised with *P. mirabilis* were also co-colonised with at least one other resistant gram-negative bacteria (range 1-4 species, median 2 species). Of the 15 patients with

a resistant P. *mirabilis* strain only one patient spontaneously cleared the organism compared to 30 to 75% clearance for other bacterial species (clearance of other species *P. mirabilis*; P = 0.007, log rank), demonstrating *P. mirabilis'* ability to cause more persistent colonisation compared to other gram-negative species.[296] Of the 15 P. mirabilis strains recovered, 13 were genetically distinct demonstrating the heterogeneity of *P. mirabilis* populations between patients within the same healthcare facility.[296]

5.6.2 *Proteus* in Gastroenteritis

Proteus species may be associated with infectious gastroenteritis. In a comparison of 1271 patients with diarrhea with 1422 healthy controls *P. mirabilis* was more prevalent in patients with diarrhea (10.8% v 2.7%, P <0.001). However this study did not take into account possible administration of antibiotics to affected patients or the potential bystander or overgrowth effect.[195] *P. mirabilis* has also been associated with food-borne gastroenteritis in an outbreak in Beijing associated with the consumption of stewed pork.[297] Shi et al (2016) investigated genetic adaptation to the digestive tract in *P. mirabilis* species isolated from the vomit and faeces of patients obtained at the time of a food-borne outbreak.[298] When three clinical isolates were compared to four local and reference strains of *P. mirabilis* obtained from food, a healthy subject, and from two patients with urinary tract infections (including reference strains HI4320 and BB2000), there was evidence of strain-level genetic adaptation to the digestive tract.[298] All seven isolates encoded drug resistance genes, but only the three patient isolates contained digestive tract toxicity genes, including one with a complete type 4 secretion system (T4SS) not previously identified in *P. mirabilis*. Active horizontal gene acquisition has been demonstrated in *Proteus* strains, including a protein from *Yersinia enterocolitica*, a known GI pathogen and member of the *Enterobacteriaceae* family.[298] In summary, *Proteus* species have been linked to diarrhoeal states, but their primary pathogenic role has not been confirmed.

5.6.3 *Proteus* in the Upper Gastrointestinal Tract

Colonisation of the upper gastrointestinal tract, including the oesophagus and stomach, by *Proteus* species has been reported in infants and older adults, often associated with instrumentation of the oropharynx.[299-303] In 13 infants with feeding tubes without gastrointestinal symptoms *Proteus* was isolated from the throat in 8%, gastric juice in 15%, and duodenal fluid in 8% of patients.[300] In elderly patients with nasogastric feeding tubes (NGT), *Proteus* species were isolated from the oropharynx in 24% and

gastric fluids in 26%.[302] In another study, colonisation of the oropharynx with *Proteus* species was present in 13% of percutaneous endoscopic gastrostomy (PEG) and 21% of nasogastric tube (NGT) patients, and in the stomach in 4% and 23% of the same patients respectively.[301]

5.6.4 Association with Hepato-biliary Disease

Early culture based surveys of patients undergoing biliary surgery showed occasional isolation of *Proteus* species from the biliary tract (13% of bile samples).[304] P. mirabilis has also been recovered from bile obtained during endoscopic retrograde cholangio-pancreatography (ERCP) in 6% of cultured samples.[305]

In a metagenomic analysis using multitag pyrosequencing, the recto-sigmoid mucosal community of healthy individuals was compared with that from patients with cirrhosis. Patients with cirrhosis had an elevated proportion of *Proteus* species when compared with controls (% abundance: healthy 0.0% v Cirrhosis 0.1%, P <0.00001).[306] Urease producing microbes such as *Proteus* spp. in the gut are known to contribute to the pathogenesis of hepatic encephalopathy through the breakdown of urea to ammonia and carbonic acid.[306, 307]

In a series of patients undergoing liver resection, *Proteus vulgaris* bacteraemia was identified in two and polymicrobial infections in eight patients.[308]

5.6.5 Pancreatic Disease

There are isolated reports of *Proteus* spp. infections of the pancreas including a patient with a large infected pancreatic pseudocyst compressing the common bile duct. The cystic contents were polymicrobial, including *Proteus vulgaris, Morganella morganii, Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*.[309] *Proteus* species were components of the biofilm that forms on biliary and pancreatic stents placed via ERCP in 14 of 100 stents.[310]

5.6.6 Intestinal Disease

A small study of the downstream effects of small bowel ulceration caused by non-steroidal anti-inflammatory drugs in rats identified a mixed population of *E. coli* and *P. mirabilis*. When treated with metronidazole rats were protected from ulcer development.[311] *Proteus* species were recovered from jejunal fluid in 11% of patients with small intestinal bacterial overgrowth syndrome (SIBOS), which often follows the expansion of facultative anaerobic bacterial communities.[312] Viable bacterial

translocation of *Proteus* mirabilis has been shown in a number of models such as the caecal and colonic mucosa mono-associated mice, as well as the mesenteric lymph nodes and livers.[313]

5.6.7 Crohn's Disease

Recent research has implicated *Proteus* spp. in inflammatory bowel diseases, with evidence derived both from patient based microbiome surveys and mechanistic research. Ambrose et al (1984) used culture-based techniques to compare the recovery of pathogenic gut bacteria from the ileal serosa and mesenteric lymph nodes in 45 Crohn's disease patients and 43 patients having surgery for other indications.[199] Overall, Crohn's disease cases were more likely to have pathogenic bacteria recovered from the small bowel serosa (12/45 (27%) patients versus 6/41 (15%) controls). Of the 12 patients with positive serosal cultures, 4/12 patients were positive for *Proteus* spp. (33%). Additionally, involved and uninvolved mesenteric lymph nodes were assessed, 15/45 patients (33%) with involved nodes had a positive culture and of these, 1/15 (7%) patients grew *Proteus* spp, although other species from the *Enterobacteriaceae* seem to translocate at a higher rate.[199] Eleven of 45 samples of uninvolved nodes harboured bacteria, with 1/11 (9%) positive for *Proteus* species.[199]

Two microbiome studies have linked an over-abundance of *Enterobacteriaceae*, and *Proteus* spp. to Crohn's disease. In a paediatric study, *Proteus* species comprised 3/18 (16.7%) gram-negative bacterial strains recovered from 12 Crohn's disease patients compared to the total absence of *Proteus* species recovered from patients with ulcerative colitis, indeterminate colitis, lymphonodular hyperplasia and controls. [314] A microarray based study comparing active Crohn's disease with matched healthy controls identified an overrepresentation of *Proteus* species overall, and *Proteus vulgaris* in particular, and other members of the *Proteobacteria* phylum in patients with active ileal disease requiring surgery.[164]

A number of studies have addressed the mechanisms by which *Proteus* and other *Enterobacteriaceae* may contribute to the development of inflammatory bowel diseases. A mouse model of ulcerative colitis (TRUC mouse, a T-bet $^{-/-}$ x Rag2$^{-/-}$ knockout model) was used to demonstrate that *Proteus mirabilis* and *Klebsiella pneumoniae* can elicit colitis, and that this propensity for the development of colitis can be transmitted to wild type mice via microbiome transfer.[162]

Interactions between the *Enterobacteriaceae*, which includes *Proteus* species, and fungi (*Candida tropicalis* species) have recently been implicated in the dysbiosis that characterises Crohn's disease.[157] When Crohn's patients were compared with first-degree relatives of Crohn's patients and healthy controls, bacterial dysbiosis was identified in affected patients and unaffected first-degree relatives. Crohn's Disease patients had an increased presence of *Candida tropicalis*. Complex associations between *Enterobacteriaceae* species (*Serratia marcasens, E. coli*) and *Candida tropicalis* were confirmed in laboratory studies, showing that flagellated and/or fimbriated bacteria combined with fungal hyphae to form a robust biofilm, with these three species combined forming the thickest biofilm ($P < 0.0001$). It was postulated that biofilm enriched for immune-modulatory microbial components (lipopolysaccharides, oligomannans etc) may perpetuate inflammation in dysbiotic patients through induction of pro-inflammatory cytokine responses and induction of apoptosis. *Proteus* spp. were also strongly positively correlated with the abundance of *Candida* in patients with familial Crohn's disease, ($r^2 = 0.709$, $P = <0.005$), raising the possibility that *Proteus* spp. are capable of the same interactions, although this was not demonstrated possibly due to their low abundance.[157]

Seo et al (2015) demonstrated that in the presence of intestinal injury and colonisation with P. mirabilis, a marked pro-inflammatory IL-1β response occurs, via activation of the NOD-like receptor protein-3 (NLRP3) inflammasome.[163] This occurs only in the presence of *P. mirabilis* HpmA haemolysin, which appears to induce host macrophage induction of NLRP3. Pre-existing injury or inflammation was required for the induction of NLRP3 activity and IL-1β by *P. mirabilis* (DSS colitis), as inflammatory monocytes were required, suggesting that *P. mirabilis* may act to perpetuate and accelerate pre-existing inflammation rather than induce it. *Proteus mirabilis* was as efficient at inducing IL-1β as pathogenic *Salmonella* spp., but the presence of the HpmA haemolysin was essential for induction of IL-1β. IL-1β has been shown to be associated with disease activity in IBD patients, however in this study most other anaerobic or facultative anaerobic commensals induced TNF-α expression not IL-1β. [163] When haemolysin expression was compared across P. mirabilis strains from multiple clinical sources (pyelonephritis, catheter associated and faecal isolates), faecal isolates had the highest haemolytic activity, and had significantly higher haemolytic titres than the catheter associated strain ($P = 0.001$), but not the pyelonephritis strain ($P = 0.065$).[315]

Proteus species have been associated with the post-operative recurrence of Crohn's disease by two independent groups.[153, 154] Metagenomic surveys of patients at the time

of surgery and at six and 18 months post-operatively demonstrated that patients were more likely to have disease recurrence in the presence of detectable *Proteus* genera (P = 0.008) and the absence of detectable *Faecalibacterium* (P <0.001).[154] The combination of detectable *Proteus* species and absent genus *Faecalibacterium* (< 0.1%) in the postoperative ileal biopsies was associated with an increased risk of recurrence with an odds ratio [OR] of 14 (95% CI 1.7–110; P = 0.013). Smoking, an independent risk factor for post-operative disease recurrence, was also associated with an increased presence of *Proteus* spp. (P = 0.0130).[154] In another study by Mondot et al (2016) in 20 Crohn's disease patients undergoing ileo-colonic resection the presence of a *Proteus mirabilis* OTU was predictive of recurrence at six months post-operatively.[153]

Whether the presence of *Proteus* spp. in association with post-operative recurrence is a primary pathogenic event or secondary to the disease recurrence remains to be elucidated. However the association in both studies was established prospectively and longitudinally, with predictive association, making a pathogenic role possible.

5.6.8 Other Large Intestinal Disease

Kanareykina et al (1987) obtained samples (mouth, stomach, small intestine and faeces) from 65 patients with ulcerative colitis, performed culture based enumeration and identified *Proteus mirabilis*, *Proteus vulgaris* or the closely related species *Morganella morganii* or *Providencia rettergi* (Figure 5.2) in nearly all cases.[316] In 40/65 patients, these species were recovered from more than one anatomic site. A *Proteus* spp. protein "vaccine" was then administered and resulted in a clinical improvement in moderate to severe ulcerative colitis as well as a decrease in bacterial counts. However, as no details of the vaccine composition or any objective disease activity metrics were performed, the study overall was of low quality.[316]

A recent 16S based metagenomic study of children with and without appendicitis has shown an increase in the relative abundance of 12 genera, of which *Proteus* species were the only representatives of the *Enterobacteriaceae* family (appendicitis *Proteus* relative abundance 0.015% versus unaffected relative abundance 0%, *P* = 0.028).[317]

Proteus bacteria have been implicated in the perpetuation of colonic inflammation in diversion colitis.[318] Inflammatory conditions of the bowel increase the local concentrations of inducible nitric oxide synthase, leading to high levels of nitrate that cannot be metabolised, except by the microbiota.[168] This favours the expansion of

bacteria that are able to metabolise nitrate under anaerobic conditions, leading to a survival advantage and population expansion of *Proteus* spp. and other nitrate reducing *Enterobacteriaceae*.[318]

5.6.9 Nosocomial Infections and *Proteus* spp. Complicating Gastrointestinal Disease

Proteus species, especially *P. mirabilis* and *P. vulgaris* are common causes of nosocomial opportunistic infections. Many patients with pre-existing gastrointestinal diseases are liable to secondary *Proteus* infections, often in the context of polymicrobial infections. *Proteus* species can also cause peritonitis following perforations of the gastrointestinal tract, an analysis of 383 patients with peritonitis demonstrated *Proteus* species in 87 of 383 (23%).[217]

Proteus species can colonise medical devices placed in the gastrointestinal tract, including ventriculoperitoneal shunts[319], nasogastric tubes[301, 302], biliary and pancreatic stents[310] and trachea-oesophageal voice prostheses.[320] *Proteus* bacteria have been shown to be contaminants of gastroscopes and colonoscopes after insufficient disinfection.[200] Infections can also be acquired in the hospital setting due to environmental contamination, with *P. vulgaris* persisting on dry, hard surfaces for up to 2 days.[321] There are of reports of hospital based and community epidemics of infection with person-to-person spread, with most patients acquiring gastrointestinal carriage prior to infection.[234, 322]

5.7 Treatment

Given the extensive antibiotic resistance profiles of some *Proteus* species, antibiotic susceptibility testing should be performed in clinical cases of *Proteus* infections.

In the context of hepatic encephalopathy, rifamixin in sub-inhibitory concentrations has been found to inhibit the production of urease by *P. mirabilis.* Low concentrations of rifamixin have also been shown to decrease production of virulence factors such as haemolysins and proteases in other *Enterobacteriaceae.*[323]

5.8 Conclusions

Proteus species are hardy, adaptable and potentially pathogenic residents of the human gastrointestinal tract, and may have been under-appreciated as a cause of gastrointestinal disease. Host-microbe and microbe-microbe interactions by *Proteus* spp., and the pathogenicity of this genus that may result from population expansion in

response to environmental changes, are emerging as important aspects of disease associated with this genus.

Proteus species may play a role in inflammatory bowel disease, through direct action of the bacteria, compounded by host immune evasion and perturbation. As a gram-negative organism, *Proteus* species are intrinsically pro-inflammatory as a result of the production of lipopolysaccharide (LPS) and immune-stimulatory flagellin proteins. There may be an association between *Proteus* species and inflammatory bowel disease, especially Crohn's Disease, mainly through population expansion and immune activation. Their low population abundance does not preclude a potential large pathogenic effect. This low abundance (<2% relative abundance) pathogenicity is seen with other intestinal pathogens and subsequent illnesses such as *Clostridium difficile and* colitis, and enterotoxigenic *Bacteroides fragilis* and colon cancer.[324]

Genetic characterisation of enteric isolates as compared to urinary tract isolates will be important in determining the effect of virulence factors on gastrointestinal pathogenesis. It is possible that environmental selection pressure may influence the differential expression of virulence factors in the gut versus the urinary tract.

Research on the gut microbiome as an ecosystem is informing our understanding of *Proteus* species, yet there are still unanswered questions. These include obtaining confirmation that *Proteus* species can swarm within the human gut, and addressing the effect of individual environmental changes (e.g. surgery, pH, oxygen concentrations) on the mucosa-associated *Proteus* population. Further work will continue to clarify both the bacterial adaptations to gastrointestinal tract colonisation, and the contribution of *Proteus* to gastrointestinal disease. Refined microbiological techniques also allow identification of low-abundance species such as *Proteus*, and allow better elucidation of their possible pathogenic role.

6 Summary and Conclusions

6.1 Summary

This work has investigated the contribution of both the gut microbiome (including a putative pathogen), and the host response to the aetiology of post-operative Crohn's disease recurrence. The contribution of serologic immune responses to the diagnosis and prediction of post-operative disease has for the first time been prospectively tested in a large cohort. Using 16S metagenomics, I have addressed the possible contributing changes in the gut microbiome using a hierarchical clustering methodology to describe high-level shifts in bacterial families following surgery and in patients with disease recurrence. Finally, following on from previous work, I have performed an in-depth assessment of the *Proteus* genus and its potential contribution to gastrointestinal diseases.

6.1.1 Serologic antibodies in relation to outcome in Post-Operative Crohn's Disease

Prior to this work, the use of serologic markers for the prediction or diagnosis of endoscopic recurrence had not been prospectively tested. Using a commercially available panel, encompassing a wide range of markers, I have investigated the largest prospective post-operative cohort in the literature.[325]

As this antibody panel is not commercially available in Australia, there has previously been no comprehensive assessment of these markers in the Australian Crohn's disease population. My work identified that in patients requiring surgery, rates of ASCA IgA positivity (99% of the population) were higher than other reported cohorts. This may reflect the differences in this cohort of patients having surgery, when compared to other population based cohorts from other countries.[325] 59% of patients were positive for anti-Omp-C, which was associated with the risk of multiple surgeries.[325]

Association of serologic markers with phenotypic characteristics related to the risk of surgery were investigated. Anti-OmpC antibody positivity was linked to the risk of repeated surgery as well as failure of medical therapy prior, while anti-A4-Fla2 positivity was associated with lower risk of repeated surgery, but positivity was associated with obstructive indications for surgery.[325] Additionally, pANCA positivity was associated with inflammatory disease (B1), and negatively associated with penetrating disease. [325]

There is a dearth of literature addressing the repeated measurement of serologic antibodies, with most studies utilising a cross sectional design. My work specifically addresses both the fluctuation in positivity and the change in responses over time. Interestingly, while there is a statistically significant fluctuation in antibody titres over time, the magnitude of this change is small.[325] This indicates that a single test (as opposed to longitudinal measurement) is sufficient for risk prediction.

6.1.1.1 Diagnosis and Prediction of Recurrence

The only antibody that was associated with the development of post-operative recurrence at 18 months was anti-Fla-X, but only significantly so at baseline or 12 months. At the six and 18 month timepoints, patients with recurrence were more likely to be positive but this was not statistically significant. However, overall anti-bacterial antibody positivity (positivity for any of the four antibacterial antibodies anti-CBir1, anti-OmpC, anti-A4-Fla2 and anti-Fla-X) was significant, and patients positive for all four had an increased risk of disease recurrence when compared to patients who were negative for all. Furthermore, patients without positivity to any of these antibodies were far more likely to remain in remission.

While the quartile sum score methodology for serologic analysis has been used extensively in experimental serologic research, we were not able to show that it was a reliable predictor of post-operative recurrence. In fact, the basic positive/negative analysis (using total number of positive antibodies) was more revealing when measured at baseline, and the number positive antibody titres was associated with disease recurrence at 18 months. When adjusted for smoking, clinical risk factors and pANCA status, the adjusted OR for the number of positive markers was 1.40 (P = 0.013).[325] This indicates that positivity for microbial serologic markers (as opposed to the magnitude of the response in EU/ml) is more predictive of the risk of disease recurrence. AUROC analysis also indicated that the individual markers performed poorly for prediction of outcomes at 18 months.

One of the more interesting aspects of this work links smoking habit with antibody levels. My work has demonstrated that, in Crohn's Disease patients, smokers have lower antibody titres than non-smokers, and that past smokers have similar antibody responses to current smokers. This is the first study to address this in gastroenterology using more than a single marker.[326] This is particularly relevant as both smoking and antibody responses are risk factors for complicated Crohn's disease. However, this work shows that they are not linked processes, and smoking may not contribute to that

risk by increasing immune reactivity. In fact, antibody titres in smokers should be interpreted carefully in light of this work, as the low titres in smokers (and past smokers) may be incorrectly reassuring to the clinician.

6.1.2 The Faecal Microbiome in Post-Operative Crohn's Disease

Previous work by our research group has demonstrated an association between endoscopic recurrence and a lowered abundance of *Faecalibacterium prausnitzii* (<0.1% abundance; OR 14, 95% CI 1.7-110, $P = 0.013$) and a detectable abundance of *Proteus* species (from the *Enterobacteriaceae* family) within the mucosa of the ileum (OR 13, 95% CI 1.1 -150, $P = 0.039$).[154] While the faecal microbiome results from this thesis do not replicate this finding, the trends identified at higher taxonomic levels were in line with this previous work. An increase in the abundance of the *Enterobacteriaceae* family (to which the *Proteus* genus belongs) in faecal samples was a clear risk factor for endoscopic recurrence (adjusted odds ratio 6.35, 95% CI 1.24 – 32.44; $P = 0.026$). Furthermore, the cluster associated with a reduced risk of endoscopic recurrence (faecal samples with enriched abundance of the *Lachnospiraceae* family; adjusted odds ratio 0.47, 95% CI 0.27-0.82; $P = 0.007$) demonstrated greater OTU diversity within the *Faecalibacterium* genus, indicating that diversity within this genus may have an impact on the risk of recurrence.

The ecology of the post-operative faecal microbiome in our cohort provides further evidence of the environmental perturbation that may occur peri-operatively as a result of ecologic changes and interventions such as antibiotics. The increase in the *Enterobacteriaceae* family, coupled with a lower abundance of *Lachnospiraceae* (indicated by the fact that these are defining cluster characteristics) in patients with recurrence indicates that these changes are most likely not mutually exclusive. Previous experimental work on the role of oxygen availability in the gut has provided a potential link between these changes. There are a number of factors that influence the availability of oxygen in the gut of Crohn's disease patients in the peri-operative period. These include:

- Pre-existing inflammation prior to surgery
- Exposure of the lumen to oxygen during surgery
- Antibiotics administered during and after surgery
- Depletion of butyrate producing bacteria due to factors mentioned above

These factors have been reviewed in more detail in section 1.4.1.5. However, the result of these changes is a probable increase in oxygen availability in the post-operative

period. This likely causes a reduction in the relative abundance of the obligate butyrate producing anaerobes (such as *Clostridium* clusters XIVa and IV, including the *Lachnospiraceae*), which drives a concurrent increase in oxygen availability. This likely stimulates an expansion of the *Enterobacteriaceae* in the post-operative period. Given the *Enterobacteriaceae* family contains many pathobionts that have been linked to the pathogenesis of Crohn's disease (Table 1.7), the coupling of these changes may provide the basis for a comprehensive hypothesis to explain the early development of endoscopic recurrence after surgery.

6.1.3 *Proteus* species as Gastrointestinal Pathogens – A Systematic Review

Following a comprehensive, systematic literature review of the *Proteus* genus in gastrointestinal disease, it is clear that the *Proteus* genus is a potential pathogen in the gut. Many of the virulence factors reviewed may increase the pathogenicity of *Proteus*, especially in the context of ecologic perturbation by antibiotics, inflammation or surgery. However, it is unclear from this work if these factors are limited to the *Proteus* genus, or if they may apply to the wider *Enterobacteriaceae* family. It may be that *Proteus* is just a "sentinel" genus in a much larger perturbation at family level. Members of the *Enterobacteriaceae* have been linked to the pathogenesis of Crohn's disease in many studies[103-105, 168, 184], but only recently has *Proteus* spp. been specifically mentioned.[153, 154, 162] Given the low abundance of these species in the gut[123], this is hardly surprising but this should stimulate further work in this area.

6.2 Future Work

This thesis aimed to address three main questions in the development of post-operative Crohn's Disease. Specifically:

> What is the possible diagnostic or predictive role of testing antibody responses to antigens of enteric origin in the post-operative setting?
>
> What is the contribution of the luminal gut microbiome (the faecal stream) to the development of post-operative recurrence?
>
> What are the characteristics of the *Proteus* genus that may contribute to its potential gastrointestinal pathogenicity?

Addressing these specific questions has led to the identification of a number of future studies that are required to further expand on the results presented here.

6.2.1 Serologic antibodies in relation to outcome in Post-Operative Crohn's Disease

While this work has shown some utility for the serologic markers to predict development of post-operative disease, more work remains. The link between serologic responses and the gut microbiome remains to be investigated in detail. Further work looking at specific antibodies that map to bacterial genera of interest such as the protective *Lachnospiraceae* or the pathobionts within the *Enterobacteriaceae* may assist specifically with the prediction and diagnosis of post-operative recurrence. Furthermore, work to investigate the link between smoking and antibody titers in Crohn's disease patients would be useful, as the potential utility of these tests may not be the same in smokers and past smokers compared to those who have never smoked.

6.2.2 The Faecal Microbiome in Post-Operative Crohn's Disease

This thesis has demonstrated broad shifts in two major bacterial families (*Lachnospiraceae* and *Enterobacteriaceae*) that are associated with endoscopic outcomes in post-operative Crohn's disease. However, a larger, in-depth survey of the faecal microbiome using shotgun sequencing to address functional capacity of the microbiome would greatly add to our understanding of the changes in the post-operative period. This may be especially revealing as a bloom of *Proteus* (or other members of the *Enterobacteriaceae*) could be identified from expression levels of genes associated with alternative respiratory pathways.

The long-term perturbation of alpha diversity (lowered Shannon's Diversity Index increasing over time post-operatively, Figure 4.2) seen in both faecal and mucosal microbiome surveys is of interest. This work has demonstrated that the greatest increase in diversity occurs in the 12-18 months following the initial surgery, however longer term studies are needed to determine how long this shift continues. Furthermore, given this shift has been seen in small subsets of both Crohn's and non-Crohn's post-operative patients[154, 171], a long-term comparison (greater than 2 years postoperatively) of the microbial diversity of healthy, Crohn's and ulcerative colits patients will likely find that this resolution of diversity takes much longer than previously thought.

In terms of clinical utility, a full matched survey of stool and tissue microbiomes from Crohn's patients may allow development of a non-invasive algorithm (performed on stool) that would predict mucosal disease. This would allow the development of a stool

test that could identify microbial profiles predicting or associated with disease recurrence.

An interesting outcome of this work demonstrates the peri-operative increase in the gram-negative *Enterobacteriaceae* up to six months after surgery. This implies that antibiotic treatment after surgery either contributes to this increase, or at the very least removes competing bacterial genera. Large datasets (such as those mentioned above) could be used to address the downstream effect of antibiotics on an already perturbed microbiome, in the peri-operative setting. It may be that the use of certain antibiotics in the post-operative period may require further investigation.

Lastly, more systems level analysis of multi-level cohorts, where data were obtained from multiple sources, samples and tests (e.g. serology, metabolomics, phenotyping) could be linked to look at cause and effect (in the post-operative setting) across multiple physiologic systems. The impact of the host genetic background should be also considered, as there is some evidence that genetics may impact the diversity and composition of the gut microbiome in both health and inflammatory bowel disease. [327] This integrated analysis approach offers the best chance of integrating all the contributing factors into a cohesive explanation of the aetiology of post-operative recurrence.

6.2.3 *Proteus* species as Gastrointestinal Pathogens – A Systematic Review

Identification of *Proteus* species as a possible contributor to the aetiology of Crohn's disease was an unexpected finding of smaller, earlier analysis of the POCER study cohort.[154] Following an extensive review of the literature around *Proteus* in the gastrointestinal tract, the finding is less surprising. Firstly, a wider population level survey looking at lower abundance bacterial families in a healthy population would help to determine the true, accurate abundance of *Proteus* and other members of the *Enterobacteriaceae*. The low abundance, mucosally associated genera are likely not well represented in the current microbial population level datasets, especially those originating from faecal samples due to aforementioned bioinformatic thresholds.

Additionally, a survey of mucosal samples from Crohn's patients could be undertaken to exclude regional, geographic or ethnicity effects. It would help to determine if *Proteus* spp. are present only in inflamed sections of the bowel or also present in the healthy mucosa. *Proteus* should also be surveyed in relation to other members of the *Enterobacteriaceae* family, as there is evidence that the *Enterobacteriaceae* family may have multiple members capable of contributing to the aetiology of Crohn's disease.

A potential method for clinical testing of patients for *Proteus* species during colonoscopy is the "CLO" (campylobacter-like organism) test.[328] While originally developed to identify *Helicobacter* pylori in gastric biopsies, using biopsies from inflamed bowel it may assist in identifying patients that are colonized with urease-producing bacteria for further investigations.[328]

A review should also be undertaken to address post-operative antibiotic regimes in post-operative Crohn's patients, with the aim to optimise coverage against the gram-negative enterics (mainly *Enterobacteriaceae).* This may assist with reducing any potential for population expansion as a result of changes in environmental factors post-operatively.

There is a lack of literature addressing possible strain level adaptations that may occur within the *Proteus* genome based on site of origin. A single available paper has indicated that *Proteus* strains isolated from the gastrointestinal tract may have significant differences at a genetic level when compared to strains isolated from the urinary tract. Given the fast evolutionary adaptation rates of bacteria, there may be significant differences in virulence factor genes in gastrointestinal tract adapted *Proteus* strains. There are also very few sequences from gastrointestinal *Proteus* isolates deposited in the publically available databases such as NCBI.

Mechanistic laboratory based investigations of these gastrointestinal *Proteus* isolates may include:

- Confirmation of swarming behavior in the GI tract in experimental mice models, and eventually human models (such as M-SHIME)[329]
- Confirmation of population expansion of *Enterobacteriaceae* (especially *Proteus* spp.) in the post-operative setting.
- Addressing bacterial adaptations to specific environmental changes wrought by surgery such as alterations in pH, inflammation and oxygen availability.
- Further work to characterise cellular entry by *Proteus* species, especially into gastrointestinal epithelial cells and immune cells, such as macrophages
- Extensive sequencing and annotation of genomes obtained from human gastrointestinal samples, and comparison to isolates from other sources
- Comprehensive investigations of the role (and expression of) of *Proteus* virulence factors in the gastrointestinal environment.

6.3 Conclusions

Disease recurrence after surgery for Crohn's Disease is an intractable problem that leads to increased morbidity and disability in this patient population. While detection and treatment have been greatly improved as a result of the POCER Study, more work is required to be able to accurately predict those at greatest risk.
I demonstrated that while serologic analysis is disappointing for diagnosis of recurrent disease, there is a pattern of increased sero-reactivity that appears to increase the risk of recurrence in certain patients.

I have shown that there is a microbial profile associated with recurrence that can be identified in stool. This profile (an increase in *Enterobacteriaceae*) is in keeping with previous work on this cohort indicating a possible role for *Proteus* spp. (a member of the *Enterobacteriaceae* family) in tissue from patients with post-operative recurrence. This may assist with identification of specific bacterial families linked with both recurrence and remission using non-invasive testing. It provides further experimental support for the potential role of *Proteus* species in post-operative recurrence specifically but also in the overall disease aetiology. My work exploring the literature around *Proteus* in the gastrointestinal tract adds further supporting evidence for this hypothesis.

Overall, all aspects of this research point to the need for further population level, high resolution studies of both the mucosal and luminal gastrointestinal microbiome in the post-operative Crohn's disease population. A longitudinal study of both populations with a multiple time point pre-operative component (such as two time points prior to surgery) would be potentially very revealing of the temporal shifts associated with surgery.

Integration of this data with immune parameters (such as sero-reactivity), microbial metabolomics and functional genetic data in a well phenotyped population may be especially useful in elucidating the pathogenesis of both post-operative recurrence and the pathogenesis of Crohn's Disease.

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APPENDIX 1

Abbreviations and Definitions

Active arm	Patients enrolled in the POCER study having resectional surgery, who have endoscopic assessment at 6 months and treatment adjusted according to endoscopic findings.
AIEC	Adherent Invasive E. coli
Anti-TNF	Anti- Tumor Necrosis factor α antibody therapy such as adalimumab or infliximab
ANCA	Anti-Neutrophil Cytoplasmic Antibodies
ASCA	Anti-*Saccharomyces cerevisiae* antibodies
AUC	Area Under The Curve
AZA	Azathioprine
bp	Base Pairs
CD	Crohn's Disease
CDAI	Crohn's Disease Activity Index
CI	Confidence Interval
CLO	*Campylobacter*-like organism
eCRF	Electronic Case Report Form
CRP	C-Reactive Protein
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid

E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESR	Erythrocyte Sedimentation Rate
EU/ml	Elisa Units per ml
FDR	False Discovery Rate
GEE	Generalised Estimating Equations
GI	Gastro Intestinal
HQR	High Quality Reads
IBD	Inflammatory Bowel Disease/s
LAM	Luminal-Associated Microbiota
LFC	Log Fold Change
LPS	Lipopolysaccharide
MAM	Mucosa-Associated Microbiota
NCBI	National Center for Biotechnology Information
NOD2	Nucleotide-binding Oligomerisation Domain containing 2
nt	Nucleotides
OR	Odds Ratio

OTU/OTUs	Operational Taxonomic Units
pANCA	Perinuclear Anti-Neutrophil Cytoplasmic Antibodies
PCR	Polymerase Chain Reaction
POCER	The Post-operative Crohn's Endoscopic Recurrence Study
PPA	Per-Protocol Analysis
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	Quantitative Polymerase Chain Reaction
R^2	Coefficient of Determination
RNA	Ribonucleic acid
ROC	Receiver-Operating Characteristic
RR	Relative Risk
ROS	Reactive Oxygen Species
SCFAs	Short Chain Fatty Acids
SDI	Shannon's Diversity Index
SNP	Single Nucleotide Polymorphism
Standard care arm	Patients enrolled in the POCER study having resectional surgery, who are followed routinely without serial colonoscopy. This group will have treatment decisions guided by clinical symptoms.

TLR	Toll-Like Receptors
TNF	Tumour Necrosis Factor
T-RFLP	Terminal Restriction Fragment Length Polymorphism
UC	Ulcerative Colits
V2	Variable region 2 of the Small Ribosomal Subunit from Prokaryotic cells
6MP	6-mercaptopurine
16S	Small Ribosomal Subunit from Prokaryotic cells sequenced in 16S metagenomics

APPENDIX 2

POCER Study Protocol Version 2

POCER STUDY
POST-OPERATIVE CROHN'S ENDOSCOPIC RECURRENCE STUDY

Post-Operative Crohn's Disease Endoscopic Recurrence "POCER" study:
Endoscopic Guided Therapeutic Intervention
& Determination of Cause

Trial registration number: **NCT00989560**

Principal Investigators: **Professor Michael Kamm**
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Prof. Danny Liew, Melbourne EpiCentre
Dr Chris McSweeney, CSIRO
Professor Mark Morrison, CSIRO
Dr Carl Kirkwood, Murdoch Children's Research Institute
Dr Josef Wagner, Murdoch Children's Research Institute
Date: **19/12/2011**

Version : **2.0**

This study will be conducted in compliance with the protocol, Good Clinical Practice and all other applicable regulatory requirements, including the archiving of essential documents.

Confidential Information: No use or disclosure of this protocol is permitted with-out prior written authorization from the principal investigators

SYNOPSIS

Title	**Post-Operative Crohn's Endoscopic Recurrence ("POCER") Study: Endoscopic Guided Therapeutic Intervention & Determination of Cause**
Investigators	**Professor Michael Kamm MB BS MD FRACP FRCP** St Vincent's Hospital, Departments of Medicine and Gastroenterology, Victoria Parade, Fitzroy, VIC, 3065 **Dr Peter De Cruz MB BS FRACP** St Vincent's Hospital, Departments of Medicine and Gastroenterology, Victoria Parade, Fitzroy, VIC, 3065
Study Centres	21 study centres (see Appendix 15.2 - List of participating centres).
Study Objective	To evaluate the effect of longitudinal endoscopic monitoring of the bowel anastomosis, with therapeutic intervention tailored to the severity of endoscopic disease recurrence, on: (a) endoscopic disease progression, (b) recurrent clinical symptoms, and (c) need for further surgery. To determine the efficacy of anti-TNF therapy in preventing disease progression in patients with sub-clinical endoscopic recurrence. To determine, using state-of-the-art molecular techniques, the microbiological and immunologic factors that are causative, or associated with, disease recurrence.
Study Design	Prospective, multicentre, randomized, controlled trial. Twenty one hospitals will take part. All patients will receive post-operative medication according to their risk of recurrence. Patients will then be randomised to endoscopic monitoring or treatment according to symptoms. A further historical control group consists of previously operated patients. Anti-TNF therapy is included for patients with subclinical severe endoscopic recurrence, or high risk and thiopurine intolerance. The primary endpoint is endoscopic recurrence at 18 months. Other endpoints are clinical and surgical recurrence. Faecal inflammatory markers will be assessed as a potential non-invasive surrogate marker of recurrence. Analysis includes clinical, health-economic, and quality-of-life assessments. Molecular microbiological and immunologic studies will be performed on biopsies obtained at endoscopic assessment.
Study Population	Male and female patients undergoing resectional surgery for Crohn's disease with an endoscopically accessible anastomosis by standard endoscopy will be enrolled. Recruitment on a 2:1 ratio with a 31% drop-out: **Clinical Study:** Active arm 113 patients; Standard care arm 57 patients Historical control arm 200 - 300 patients (St Vincent's Hospital only) **Mucosal bacteriology/immunology:** Crohn's Disease 30 patients; UC disease controls 10 patients; and Right sided cancer resection controls 10 patients
Study Duration	Primary end-point is at 18 months; starting August 2009 Participants may have extended follow-up over 5 years.
Evaluation methods:	**Clinical centres:** Following risk stratification all patients will be randomised to either the Active Arm (endoscopic monitoring) or Standard Care Arm (treatment based on clinical symptoms). **Tissue studies:** At St Vincent's, St Vincent's Private and The Royal Melbourne Hospitals – tissue collected for mucosal bacteriology and immunology
Statistical considerations	Categorical variables: chi-squared tests (or Fisher's Exact tests for small samples). Continuous variables will be applied to (parametric) t-tests and (non-parametric) Mann-Whitney tests for symmetrically and asymmetrically distributed data, respectively.

STUDY SCHEDULE (TABLE 1)

Study Forms/ Assessments and Recordings	Baseline	Surgical Resection	1/12	2/12	4/12	6/12	7/12	8/12	10/12	12/12	15/12	18/12	24/12	Unscheduled Visit
Study Visits – In Person	X	X	X			X				X		X		X
Study Visits – By Phone or In Person				X	X		X	X	X		X		X	
Study Eligibility and Informed Consent	X													
Contact Information and Demographics	X													
Risk stratification & randomisation	X													
Pre-Operative Medications		X												
Patient History	X													
CDAI	X					X				X		X		X
Current Medications	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Health Care Resource Utilization	X		X	X	X	X	X	X	X	X	X	X	X	X
IBDQ and SF-36	X					X				X		X		
Symptom Assessment	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Routine Blood Investigations	X		X	X	X	X	X	X	X	X	X	X		X
Study Specific Blood Tests and Study Faecal Calprotectin	X					X				X		X		
Endoscopic Assessment						X active arm only						X		+/-
Adverse Events	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Anti-TNF Safety Screening	If indicated	If indicated				If indicated							X	
Drop-out and Treatment Failure	If indicated	If indicated	If indicated	If indicated	If indicated	If indicated	If indicated	If indicated	If indicated	If indicated	If indicated	If indicated		X

Additional consultations outside the study schedule will be arranged as required and recorded in the CRF

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ABBREVIATIONS AND DEFINITIONS

Definitions and Terminology

Inflammatory bowel disease (IBD) is the name of a group of disorders that cause the intestines to become inflamed, which can lead to long term chronic disability and premature death. The main forms of IBD are Crohn's disease (CD) and Ulcerative Colitis (UC).

Resectional surgery is considered as a complete excision of Crohn's disease affected bowel with macroscopically normal margins at both ends of the diseased bowel segment, together with local lymph nodes. In Crohn's disease the most common site of disease is the terminal ileum but segments of proximal small bowel and colon may also be involved.

Anastomosis refers to the join created between two macroscopically normal portions of bowel after resectional surgery. The anastomosis is the most common site for recurrence of Crohn's disease.

Post-operative Crohn's disease "recurrence" is an umbrella term which refers to the presence of new objective intestinal lesions of CD after surgical resection. ***"Endoscopic recurrence"*** refers to the occurrence of new mucosal lesions, usually in the terminal ileum or colon that can be visualized endoscopically after surgery. "***Clinical recurrence"*** refers to the development of clinical symptoms associated with an elevated CRP or elevated platelet count. **"*Surgical recurrence"*** refers to the requirement of a further abdominal operation after the resectional surgery that led to study entry.

Study Population: Male and female patients with Crohn's disease having resectional surgery, who have an anastomosis accessible by standard colonoscopy.

Active arm: Male and female patients with Crohn's disease having resectional surgery, who have an anastomosis accessible by standard colonoscopy. These patients will have endoscopic assessment at 6 months and treatment adjusted according to endoscopic findings. This group will also have a colonoscopy at eighteen months (the primary end-point).

Standard care arm: Male and female patients with Crohn's disease having resectional surgery, who have an anastomosis accessible by standard colonoscopy who are followed routinely without serial colonoscopy. This group will have treatment decisions guided by clinical symptoms. This group will have a colonoscopy at eighteen months (the primary end-point).

Historical controls: Male and female patients with Crohn's disease who have had previous surgery and have been managed by standard care, in whom the outcomes are already known. This will allow comparison between patients with different durations since surgery.

Healthy tissue controls: Male and female patients without Crohn's disease having a colonoscopy for rectal bleeding or other risk factors for cancer.

Healthy tissue controls from patients with previous resection: Male and female patients without Crohn's disease who have had a previous right hemi-colectomy for right sided colon cancer who are having a colonoscopy for cancer surveillance.

Diseased tissue controls: Male and female patients with ulcerative colitis under-going colonoscopy for disease assessment or surveillance.

Emergency operation: Any operation occurring as a result of a bowel obstruction or free perforation that does not settle with conservative management and warrants surgery within the same admission.

ABBREVIATIONS AND DEFINITIONS (continued)

CD	**Crohn's Disease**
CRF	**Case Report Form**
e-CRF	**electronic Case Report Form**

Serious Adverse Event

Any event that:

Results in death
Is life threatening
Is a persistent or significant disability/incapacity
Requires inpatient admission
Prolongs hospitalisation
Is a congenital abnormality or birth defect
Is a medically important event that requires intervention to prevent one of the outcomes above

1. INTRODUCTION AND STUDY RATIONALE

This project aims to:

Determine whether an endoscopic regime with defined therapeutic interventions diminishes recurrence in post-operative Crohn's disease (compared to current standard care);
(2) Use recent state-of-the-art molecular microbiological techniques to identify the novel unidentified bacteria that cause Crohn's disease. A newly operated bowel without mucosal disease, followed prospectively, offers the greatest opportunity to achieve both these aims.

1.1 The Clinical Problem

Eighty percent of patients with Crohn's disease undergo surgery during their life. Even if all macroscopically involved intestine is removed, 75 percent of operated patients have new lesions at the anastomosis one year after surgery.[1,2] Seventy percent of operated patients will require a second operation. Established risk factors for recurrent disease include restoration of the luminal stream, presence of an anastomosis, perforating disease and smoking.[3,4] Recurrent disease is a major source of morbidity, impaired quality of life, and cost.[1,2] The total annual cost of inflammatory bowel disease in Australia is $2.7billion per year.[5]

The current standard of care for post-operative management involves preventive drug therapy for those at greatest risk, but no ongoing assessment for disease recurrence unless symptoms develop.

Recent studies in active Crohn's disease have demonstrated clearly that mucosal healing is associated with a substantially decreased rate of clinical symptoms and need for hospitalisation and further surgery. In the post-operative setting, after the removal of diseased intestine, it is well established that mucosal disease recurrence can be observed endoscopically before symptoms develop. The small number of drug trials that have assessed the ability to reduce post-operative recurrence have therefore focused on endoscopic and clinical recurrence. Even the best proven strategy of 3 months treatment with metronidazole combined with one year azathioprine therapy in patients with one or more risk factors for recurrence produces modest benefit in terms of a decreased incidence of severe endoscopic recurrence. [6,7] Anti-TNF therapy has recently been shown to decrease post-operative recurrence when applied to all patients post-operatively, but has not been administered selectively to those at highest risk or those in whom a standard immunosuppressive drug such as azathioprine has failed.[8] The targeted use of anti-TNF therapy in patients with sub-clinical mucosal recurrence has not been studied previously.

No study has (1) included patients at different risk of recurrence, and then stratified therapy accordingly; or (2) provided a clinical framework to monitor recurrence and adjust therapy if the disease recurs or progresses.

To our knowledge only one study, published in abstract, has addressed the value of post-operative endoscopic monitoring and adjustment of treatment according to endoscopic findings.[9] In this retrospective study 90 patients with an endoscopically accessible anastomosis were colonoscoped within 12 months of surgery, while 42 controls were not. Those with severe endoscopic recurrence were treated with immunosuppressive therapy. Clinical recurrence was significantly greater in those who were not colonoscoped after surgery.

We intend using published controlled trials as the basis of a therapeutic management study for newly-operated patients with Crohn's disease, but incorporating risk stratification, endoscopic monitoring to detect early disease recurrence, and tailoring therapy according to the endoscopic findings. We believe such a strategy has the potential to decrease morbidity, and to decrease the economic burden, associated with recurrent disease.

We believe this study has the potential to alter the current standard of care, which is ad hoc, empiric and symptom-based, to one which is preventive, and endoscopically-based

with structured interventions. If effective, this will diminish disease recurrence and its sequelae.

1.2 The Cause of Crohn's Disease and its Recurrence

We wish to establish the factors associated with recurrence of Crohn's disease. We believe that integration of two state-of-the-art molecular microbiological techniques, with microbiota studied over time from a starting point of absent mucosal disease at a site of known recurrence, offers the best chance of finding the microbiological cause of Crohn's disease, akin to the Helicobacter of peptic ulcer disease.

CD is characterized by an immunological response to gut microbiota occurring in genetically susceptible individuals.[10]

There is strong evidence for involvement of microbes in CD. Diverting the faecal stream from inflamed gut causes healing, and re-infusion of intestinal contents into surgically excluded ileum triggers recurrence.[3,11-14] Antibiotics modify post-operative recurrence. Incomplete concordance in monozygotic twins also suggests an important role for an environmental factor.[15] Animal models of inflammation require bacterial flora to induce inflammation.[16 17]

Identifying the microbiota implicated in CD has focused on identification of novel pathogens or changes in gut microbial composition [18,16,19-21] and diversity. No definitive causative data have emerged, but identified changes include: increased *E. coli*, *enterococci*, and *Fusobacteria* post-operatively[16] and presence of *Mycobacterium avium paratuberculosis*.[22]

To our knowledge only two studies have addressed the microbiota in post-operative CD patients. Using FISH analysis a reduction in microbiota diversity, notably *Faecalibacterium prausnitzii*, was associated with greater recurrence.[23] Adherent invasive *E. coli* was isolated from 65% of ileal resections & 36% of biopsies of early neo-terminal ileal lesions[24,25,26]

Traditional culture methods are extremely limited and can identify only a small proportion of the organisms present in the gut. Molecular microbiological techniques offer a huge advance in identification, including major whole species, but the most recent high-throughput techniques have not yet been applied in this novel setting. [27-30]

Two techniques that examine the gut microbial community have revolutionized the ability to identify novel organisms in the gut. These two techniques are complementary: microarray technology is fast, cheap, applicable to all samples, and an excellent screening tool, while pyrosequencing provides a more detailed analysis and needs to be restricted to a subgroup of patients due to its labour-intensive nature and expense. These techniques have not been applied previously in this setting.

(1) Microarray analysis of gut microbial community

An initial assessment involves using a phylogenetic custom microarray composed of small subunit (SSU) ribosomal DNA (rDNA) probes (around 800 unique sequences) for analysis of gut microbial diversity that has been developed, optimized and validated by the CSIRO using healthy human, Crohn's disease and animal faecal samples. The DNA probes derive from microbial 16S rRNA gene sequences from the human gut deposited in public databases, and enable identification of the diverse human gut microbiota as currently understood. Specificity and sensitivity have been validated using DNA from a known cohort of patients with IBD, and shifts in microbial populations observed by microarray analysis confirmed by quantitative real time PCR assays.

This technique allows batch testing of samples, and comparison of microbial diversity at different time points. The microarray has been validated on biopsies taken for mucosa-associated microbiota.[31]

(2) Pyrosequencing

A more detailed investigation is undertaken using pyrosequencing, which detects known and novel phyla within the microbiome, and allows monitoring of microbiota population dynamics. It also uses 16S fingerprinting. Four enzyme DNA sequencing technology monitors the real-time DNA synthesis detected by bioluminescence. It enables high throughput sequencing of hundreds of base pair reads.[32] It is complimentary to the microarray, allowing deep sequence analysis of the microbiome and the ability to define microbiota down to the genera level. In addition genera specific quantitative real-time PCR can be employed to measure discrete populations of microorganisms.

1.3 Study Hypotheses

That a prospective endoscopically-guided management programme, with pre-determined therapeutic interventions, will lessen the severity of disease recurrence, health care resource use, and the need for further surgery in patients operated on for Crohn's disease.

That specific changes in gut mucosal micro-flora at a Crohn's anastomosis cause disease recurrence.

That early changes in immune cell function / activity reflect sensitization to microbial flora, and that this activation differs in those with recurrence from those without recurrence.

That a reproducible, validated endoscopic scoring system will enhance post-operative management and will serve as a useful tool in studies of prevention of post-operative recurrence.

That anti-TNF therapy will lessen recurrent disease severity in patients resistant to, or intolerant of, standard immunosuppressive therapy.

That faecal calprotectin is a reliable bio-marker of disease recurrence.

2. STUDY AIMS AND OBJECTIVES

The primary aim is to evaluate the effect of longitudinal endoscopic monitoring of the bowel anastomosis, with therapeutic intervention tailored to the severity of endoscopic disease recurrence, on: (a) endoscopic disease progression, (b) recurrent clinical symptoms, and (c) need for further surgery.

To prospectively characterise endoscopic, histologic, microbiological and immunologic factors that are associated with disease recurrence at the anastomosis in patients having resectional surgery for Crohn's disease.

To examine the benefit of anti-TNF therapy in modifying disease recurrence in patients with high risk of recurrence or patients who have failed standard immunosuppressive therapy.

To establish a reproducible scoring system for evaluating post-operative disease recurrence.

To evaluate the cost-benefit of endoscopic disease monitoring in post-operative Crohn's disease patients

To assess faecal calprotectin as a noninvasive measure of disease recurrence.

3. STUDY DESIGN

3.1 Clinical Study – Preventing Disease Recurrence

This is a prospective, multicenter, randomized controlled trial designed to test, ***for the first time***, the value of endoscopically-guided intervention in post-operative Crohn's disease patients. We have formulated a prospective diagnostic and treatment algorithm (See Figure 1).

There will be three arms to the clinical study. Post-operative patients will be randomized to (i) ***an "active" arm***, who have endoscopic assessment at 6 months and treatment adjusted according to endoscopic findings, or (ii) a ***parallel control arm ("standard care"),*** in which patients are stratified in the same way to initial drug treatment according to risk, but do not have an endoscopy at 6 months and have treatment changes only if clinically indicated. Assessment of both "active" and "standard care" arms at 18 months include clinical, surgical recurrence, and endoscopic recurrence.

A third arm consists of a ***historical control group***, consisting of patients who have had previous surgery and have been managed by standard care, in whom the outcomes are already known.

This study employs the current best test, endoscopic surveillance, to detect and monitor mucosal disease recurrence. However, preliminary studies suggest that stool inflammatory markers, especially calprotectin, are raised in active disease.[33] We will therefore test stool prior to each endoscopy, to determine whether faecal testing is a reliable surrogate marker for endoscopically-identified inflammation.

Health-economic and quality-of-life benefits of an endoscopic-based drug intervention strategy, compared to standard care, will also be assessed. Health-economic as well as generic and disease-specific quality-of-life measures will be employed.

3.2 Drug Treatment

Post-operatively all patients receive three months metronidazole at dose of 400mg bd. High risk patients are additionally placed on a thiopurine. Patients are then randomised to be endoscoped at 6 months, or managed clinically.

If the 400mg bd dose of metronidazole is poorly tolerated it can be reduced to 200mg bd and if still poorly tolerated can be further dose reduced to 200mg daily. Antibiotics for a non-Crohn's indication, such as chest infection or UTI, can be prescribed for up to six weeks continuously. Antibiotics prescribed outside of those stated in the protocol for a Crohn's related indication (e.g. active perianal disease) must be discussed with the principal investigators prior to commencement. Patients requiring multiple courses of antibiotics for other indications during the study must be discussed with the principal investigators.

Patients on azathioprine pre-operatively remain on this drug if considered high risk, at a dose of 2mg/kg/day. Patients who do not tolerate azathioprine will receive mercaptopurine in a dose of 1.5mg/kg/day. Patients must be commenced immediately on the full study dosage of the thiopurine; upward titration over multiple visits is not permitted. If the TMPT level is known to be low at study commencement then an adjusted lower dose of thiopurine is permitted. Patients on azathioprine or mercaptopurine must have a full blood count and liver function tests 2 and 4 weeks after starting therapy, monthly after that for three months and as clinically indicated for the remainder of the study. The dose may need to be adjusted if blood test abnormalities are detected, however the direct measurement of TPMT activity or thiopurine metabolites may not be performed at any stage during the study. Dose of either drug may only be adjusted down if there is a total white cell count < 4 or abnormal liver function at any stage. Azathioprine and mercaptopurine may only be adjusted to a higher dose on the basis of a patient's weight. Dose reductions of any study medications that are poorly tolerated must be recorded as an adverse event.

If either azathioprine or mercaptopurine are not tolerated, or associated with an adverse event, cease these drugs. This should be determined no later than four weeks after the operation; the patient may then commence antibody therapy within 7 days of completion of screening procedure.

Those intolerant of thiopurine before surgery who are at high risk will receive antibody therapy post-operatively. Patients on antibody therapy pre-operatively who are deemed low risk must cease anti-TNF therapy at study commencement. Any steroids prescribed for Crohn's Disease (e.g. Prednisolone) taken in the pre-operative period must be tapered within four months of surgery.

Patients may enter the study taking a thiopurine and allopurinol (following past thiopurine metabolite testing), provided the dosage of drug has been stable for 3 months.

All study drugs (except adalimumab) should be commenced within seven days post-operatively. Commencement or alteration of study drugs outside of this time period must be discussed with the principal investigators.

At 6 months endoscopy, those with a Rutgeert's score on endoscopy of grade i2 or more will have a step up in therapy. Those high risk patients on thiopurine will step up to standard antibody therapy. Those already on fortnightly (standard) 40milligram adalimumab will increase to weekly 40milligram adalimumab.

In high risk patients who have not tolerated a thiopurine, or high risk patients who develop severe active endoscopic disease despite adequate thiopurine treatment, we will use the anti-TNF antibody, adalimumab (Humira). Adalimumab will be administered in a standard dose: 160mg initially, 80mg two weeks later, then 40mg per fortnight continuing. Prior to use of adalimumab participants will undergo routine screening for Tuberculosis with history, chest X-ray, QuantiFeron Gold as well as ANA, dsDNA, Hepatitis B and C. High risk patients who are tolerating a thiopurine at 6 months and require treatment intensification with the anti-TNF antibody adalimumab will continue the thiopurine after the commencement of adalimumab.

At 6 months endoscopy, those low risk patients with a Rutgeert's score on endoscopy of grade i2 or more will step up to treatment with either azathioprine at a dose of 2mg/kg/day or mercaptopurine at a dose of 1.5mg/kg/day. If treatment with either of these drugs is not tolerated, the patient will be switched to treatment with standard doses of Adalimumab as described above.

Adalimumab will be provided as supplied study drug for the treatment of high risk thiopurine intolerant patients from baseline, and for low risk (thiopurine intolerant) and high risk (thiopurine tolerant) high risk active arm patients with endoscopic recurrence >i2 at 6 months. Adalimumab will be provided for the study duration, until the 18 month endpoint only.

Treatment with loperamide or cholestyramine, or mild analgesia such as paracetamol (not non-steroidal drugs or opioids, apart from in the immediate post-operative period) is permitted in the study, and will not of their own necessitate study withdrawal.

Patients should be counseled to avoid taking non-steroidal anti-inflammatories (e.g. ibuprofen, diclofenac) while participating in the POCER Study. Patients requiring medications with activity against Crohn's disease for another indication (such as steroids for Rheumatoid Arthritis) must withdraw from the study.

Patients must also be informed not to change, add or alter the dosages of any (study or concomitant) medications without consultation with the study site. All alterations to (study or concomitant) medications must be recorded on the eCRF.

Patients and/or treating clinicians must not cease any study drugs at any stage without prior notification of the principal investigators.

3.3 Drop Out and Treatment Failure

Patients can drop out of the study at any time if they wish. For the purpose of analysis these patients will be included as last observation carried forward and intention to treat.

Patients in either arm who develop clinical symptoms, necessitating either an unscheduled colonoscopy or change in anti-inflammatory drug treatment, will be withdrawn from the study, and will be included in the analysis as failures (intention to treat analysis). In the event of an unscheduled colonoscopy, the site investigator must complete the endoscopic assessment form.

Patients who meet the eligibility criteria for the study after their initial resection may require subsequent reoperation or creation of a stoma as a consequence of post operative complications (such as an anastomotic leak). These patients will be required to withdraw from the study and will be considered withdrawn prior to treatment if the intervention occurs within the post-operative hospital stay. These patients may be eligible to re-enter the study on closure of stoma.

Adalimumab will not be used for patients who develop clinical symptoms consistent with clinical recurrence of Crohn's during the study; patients who develop clinical recurrence will drop out of the study (and can then access other therapies, such as anti-TNF therapy, as part of their routine clinical care (using PBS criteria, etc). Adalimumab will only be used: (1) in high risk patients who are intolerant of thiopurines prior to or within four weeks of their operation, (2) in high risk patients who have Rutgeert's ≥ 2 endoscopic recurrence at their six month colonoscopy, and (3) in low risk, thiopurine intolerant patients who have a Rutgeert's ≥ 2 endoscopic recurrence at their six month colonoscopy. .

3.4 Study Timeline

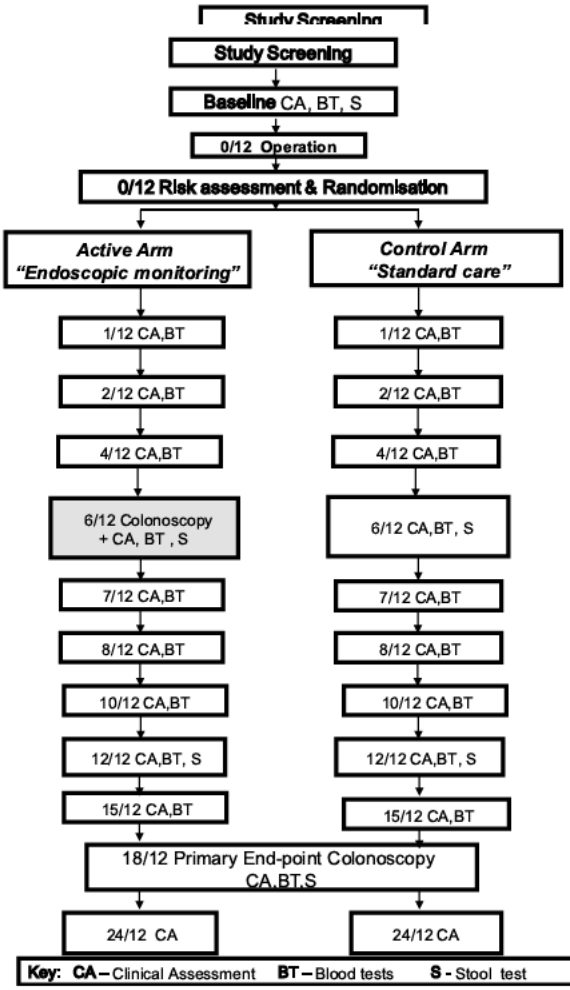


Figure 1. The active arm of the study is shown below, detailing the timing of endoscopy, stratification after
3 months open-label metronidazole treatment according to risk of recurrence, and treatment
actions according to endoscopic findings.

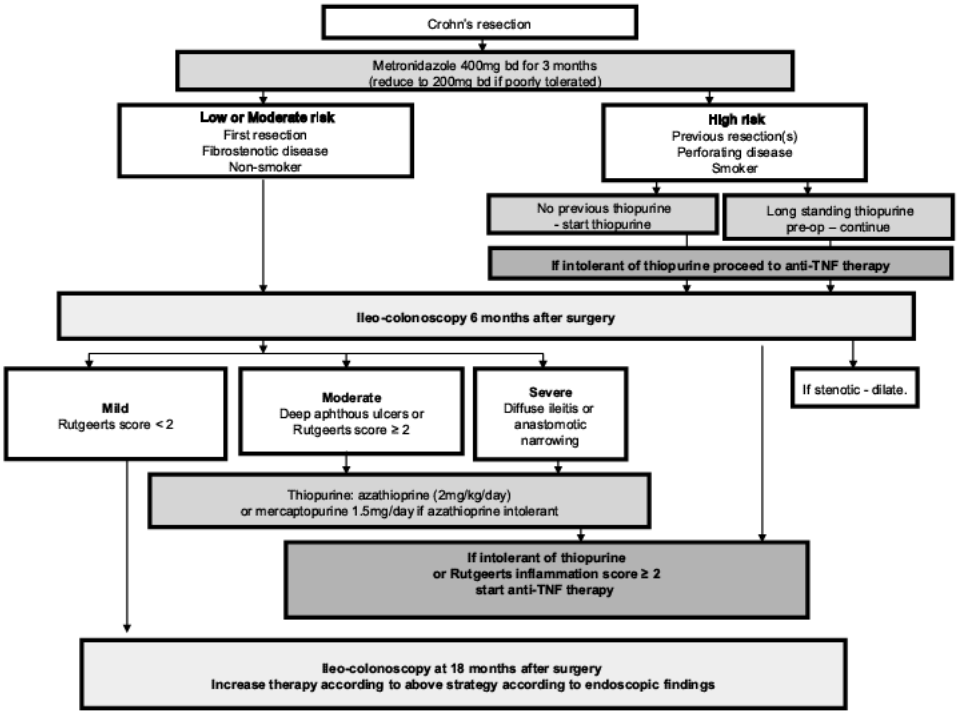
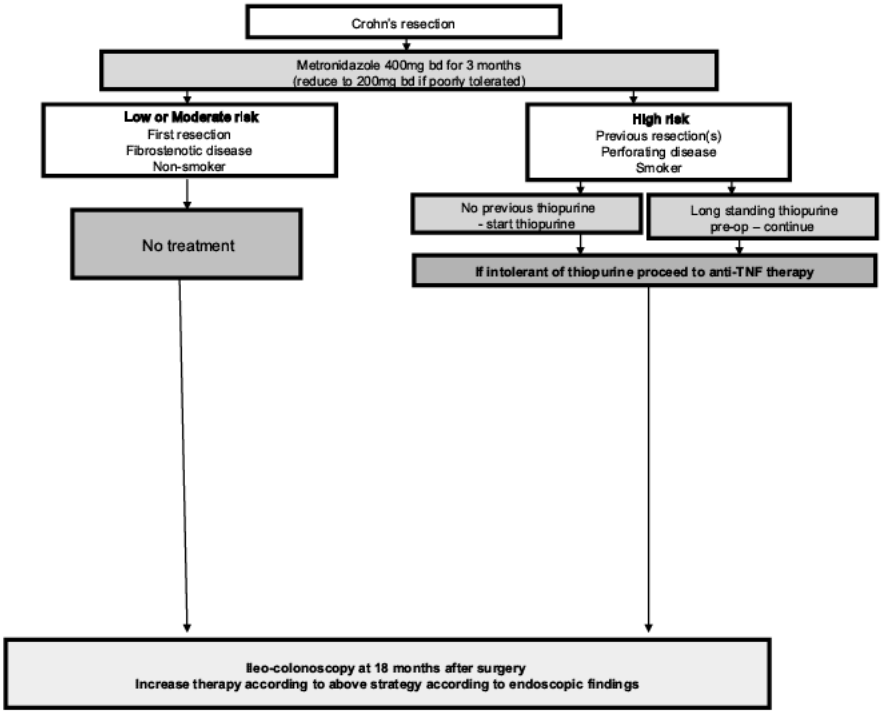


Figure 2. The standard care arm is shown below and consists of standard clinical care with ileo-colonoscopy at 18 months.



3.5 Study Plan

Patients undergoing intestinal surgery for Crohn's disease with an endoscopically accessible anastomosis will be enrolled. Patients may be enrolled in the study up to two weeks post operatively. Any patient who has undergone a recent resection for Crohn's disease outside of the defined recruitment period may be recruited to the study at the discretion of the principal investigators. All patients will be assessed for their risk of recurrence, and drug treatment provided according to the criteria below.

High Risk
One or more of the following:
Patient is a current smoker
Patient has had a previous bowel resection for Crohn's Disease
Patient has perforating disease (abscess, perforation or fistula previously or now) including perianal disease
Low Risk
None of the above

Table 2. Criteria for risk stratification.

In patients who are being enrolled following a reversal of stoma, the operations to create and reverse the stoma are considered together as one operation. Therefore, the patient must still meet one or more of the above criteria (such as an additional previous bowel resection) to be stratified high risk.

Patients will then be randomized post-operatively to either (i) undergo endoscopic monitoring at 6 months post-operatively and treatment adjustment accordingly ("active arm") or (ii) will receive standard clinical care, without scheduled colonoscopy, based on treatment according to clinical symptoms ("standard care arm").

Within study centres patients will be randomized to "active arm" and "standard care" arms. The latter serves as one control group for the active arm.

A second control group for clinical and surgical outcomes will be a retrospective cohort of patients operated up to five years previously ("historical controls").

Patients will be assessed pre-operatively and post-operatively according to the study schedule in table 1 for: (1) clinical state, (2) blood tests, and (3) stool collection.

Clinical symptoms will not form an indication for colonoscopy or change in treatment, unless there is clinical recurrence defined by the presence of clinical symptoms together with changes in laboratory tests of an elevated CRP or platelet count. If there is clinical recurrence and the investigator elects to colonoscope the patient or changes the patient's treatment then the patient is deemed a treatment failure and drops out of the study.

All patients will have a colonoscopy at 18 months (primary endpoint).

For all patients having a scheduled colonoscopy, the date of colonoscopy must be within 7 days either side of the dates generated by the e-CRF for the six month review (active arm only) and eighteen month review (active and standard care arms).

4. STUDY ENDPOINTS

4.1 Clinical Outcomes

Co - Primary endpoints: ***(i)*** **Endoscopic recurrence and (ii) severe endoscopic recurrence at 18 months post-operatively.** Recurrence defined as the presence of any aphthous ulcers, and severe recurrence defined as an endoscopic Rutgeerts score ≥ 2 (5 aphthous ulcers or larger ulcers confined to the anastomosis). [7]

Secondary endpoints: (1) **Clinical recurrence at 18 months**, defined as recurrence of symptoms consistent with recurrent disease, necessitating new treatment, or Crohn's Disease Activity Index (CDAI) > 150; (2) **Surgical recurrence at 18 months,** defined as the need for further intestinal surgery due to Crohn's disease (3) **Health care resource use**; (4) **Quality of life** measured prospectively using the disease specific IBDQ and the generic SF-36.[34,35]

Treatment Failure (Clinical Recurrence): Number of patients who drop out of the study because of clinical symptoms, associated with confirmed disease recurrence necessitating addition of new drug therapy prior to the 18 month post-operative date.

4.2 Scientific Outcome

To identify changes in the microbiota at the anastomosis associated with disease recurrence, progression, and more severe disease.

4.3 Outcomes and Significance

First prospective study of endoscopic monitoring and therapeutic intervention.
First prospective evaluation using molecular techniques to identify the microbiological changes occurring at the Crohn's anastomosis.
Identification of risk factors for endoscopic and clinical recurrence.
Demonstrate improved post-operative management (decreased recurrence).
First evaluation of anti-TNF therapy for recurrent mucosal, subclinical disease.
First prospective endoscopically-validated evaluation of faecal calprotectin as a marker of recurrent disease.

5. STUDY POPULATION

5.1 Inclusion criteria

All patients with Crohn's disease who undergo resection with an endoscopically accessible primary anastomosis which results in macroscopic normality.

Patients having a reversal of an temporary ileostomy created after previous surgery for Crohn's disease may be enrolled provided that the reversal of the ileostomy results in a primary anastomosis and macroscopic normality of the remaining bowel.

Patients with co-existing perianal disease may be included provided the resection has led to a primary anastomosis and macroscopic normality of the intestine.

Patients must have proven history of Crohn's disease based on (clinical, radiologic, endoscopic and histologic criteria).

5.2 Exclusion Criteria

Patients with anastomosis which is endoscopically inaccessible by standard colonoscopy

Patients in whom there is persisting macroscopic abnormality post surgical resection.

Patients with Crohn's disease who have an end stoma (ileostomy or colostomy)

Patients for whom endoscopy is not suitable due to co-morbidities or unwell clinical state

Inability to give informed consent.

Inability to obtain access to the anastomosis at colonoscopy.

Suspected perforation of the gastrointestinal tract.

Pregnancy

5.3 Randomisation of subjects

The electronic CRF (e-CRF) will randomise patients to either the "active arm" or the "standard care arm". Patients will be randomised in blocks of three in a 2:1 ratio of two patients randomised to "active arm" and one randomised to "standard care arm". This will ensure that each study site will have an equal distribution of two "active arm" patients for every one "standard care" arm patient.

5.4 Number of subjects

Sample size calculation is based on: alpha value = 0.05 (1-sided); (2) power = 80%; (3) expected rate of the primary outcome (defined above) at 18 months for control group = 60%; and (4) expected rates of the primary outcome (defined above) at 18 months for active group = 35%. These expected rates are based on (1) the randomized, placebo-controlled trial of metronidazole with azathioprine to prevent postoperative recurrence of Crohn's disease [36], which demonstrated that at 12 months post-operatively, "significant" endoscopic recurrence occurred in 69% of the metronidazole-only arm and 44% of the metronidazole-plus-azathioprine arm (per protocol analysis, p=0.048); and (2) the randomized study of infliximab versus placebo in post-operative Crohn's disease, which demonstrated that at 12 months post-operatively, severe endoscopic recurrence occurred in 54% of the placebo arm and 9% of the infliximab arm [8].

For recruitment on a 2:1 ratio, 84 "active group" participants (endoscopic monitoring group) and 42 "standard care" controls are required. To allow for a 31% drop-out of subjects, we will recruit 113 and 57 patients in both groups respectively. There will be sufficient patients undergoing surgery in the participating centres to achieve these numbers.

Therefore:

Active arm 113 patients
Standard care arm 57 patients
Historical control arm > 200 patients

Total study enrolment 170

Laboratory studies (subgroup of the clinical): Mucosal bacteriology and immunology

Based on practical ability to process tissue in the study time-frame, and previous studies undertaking similar molecular evaluation of tissue [23] we will undertake tissue studies on 30 patients with Crohn's disease and 20 non-diseased controls.

This is based on a randomized control trial in which Crohn's disease patients were randomized to a probiotic *Lactobacillus johnsonii* or placebo. Amongst the 13 of 21 patients who developed endoscopic recurrence (defined by a Rutgeert's score ≥ 2) at 6 months post ileal resection, endoscopic relapse was consistently associated with a lower proportion of *F. prausnitzii* isolated at the time of surgery and (*ii*) a lower proportion of Firmicutes (i.e. *C. coccoides* and *F. prausnitzii*) 6 months after surgery. [23]

Therefore:

Disease 30 patients
Non-diseased controls 20 patients

5.5 Withdrawal from study

Patients will be withdrawn from the study if any of the following occurs:

The patient requests withdrawal from the study. The patient can decide to stop their participation in the study for any reason and at any time during the study.

The patient fails to undergo surgery or have follow-up endoscopic investigation.

Treatment Failure (Clinical Recurrence): Development of clinical symptoms, associated with confirmed disease recurrence necessitating addition of new drug therapy prior to the 18 month post-operative date. Clinical recurrence defined as: symptoms typical of Crohn's disease, necessitating repeat endoscopy or addition of new drug therapy.

Any pregnancy that precludes a colonoscopy at 6 months (active arm) and/or at 18 months (all patients) will require withdrawal from the study. Any patient becoming pregnant whilst undergoing treatment with adalimumab will also be required to withdraw from the study.

The investigator can also decide to withdraw a patient from the study if he/she considers it as necessary (e.g. non-respect of at least one of the selection criteria known after inclusion).

If the patient misses any one of the major study visits at 6, 12 and 18 months.

6. EVALUATION CRITERIA

6.1 Endoscopic Visual Assessment

Assessment of the anastomosis will be made using 3 established scoring systems: (1) Crohn's Disease Endoscopic Index of Severity (CDEIS), (2) Simple Endoscopic Score for Crohn's disease (SES-CD), and (3) Rutgeerts endoscopic grading scale, the only post-operative, but unvalidated, endoscopic scoring system.[36] Using a combination of these scoring parameters we will derive and validate a new scoring system which will be called the POCER score. Site investigators must complete the endoscopic assessment form, obtain photographs as described below, and provide details of the endoscopic equipment utilized. The same site investigator (if possible) must perform both the 6 and 18 month colonoscopies if the patient has been randomized to the 'active' arm of the study.
Photographs must be taken of the anastomosis (1) close up, and (2) at a distance. An additional photograph of the pre-anastomotic segment must also be taken. This is to document: (a) the presence, number, size and depth of ulcers, (b) the presence and severity of inflammation, and (c) the presence and degree of stenosis. The anastomosis should be washed prior to photography, and photographs must be taken prior to any biopsies.
The ileo-colonic anastomosis should be selected as the study focus, except in cases of colonic (rather than ileo-colonic) resection, where the colo-colonic anastomosis should be assessed. In

the case of multiple small bowel resections where there is not an ileo-colonic anastomosis, the most distal small bowel anastomosis should be assessed.
A digital camera should be present at all study endoscopies as a contingency in the event of equipment failure.

Photographs must be uploaded to the eCRF within 48 hours.

All final Rutgeert's scores must be assessed and confirmed correct by the POCER principal investigators prior to any alterations to the patient's drug therapy.

6.2 Stool testing

Stool will be collected at 4 time points in the study (see table 1) and assayed for faecal inflammatory markers, other inflammatory markers, and microbiota. These measurements will not influence the medical management during the study. The pre-operative stool sample must be collected prior to the administration of any bowel preparation. Faecal specimens will be collected by the patient prior to admission for initial surgery and prior to bowel preparation/admission for follow-up colonoscopy.

Prior to admission, consented participants will be provided with a sterile vessel for collection of a stool sample at home prior to consumption of the bowel preparation. The sample will be stored briefly at -20°C (home freezer), and then transported frozen to the laboratory, where it will be stored at -80°C until analysis.

A similar number of control cases (approximately 20 cases) will be recruited from patients (1) with a different inflammatory disorder (i.e. ulcerative colitis),, and (2) who are being screened for a non-inflammatory condition (i.e., colorectal cancer and/or those undergoing colonoscopic surveillance subsequent to hemi-colectomy for colonic cancer).. Tissue samples and blood and faecal specimens will also be collected from these patients.

6.3 Routine Blood testing

Routine blood tests for full blood count, urea and electrolytes, liver function tests, ESR and CRP will be performed by each hospital's local laboratory as part of routine clinical care. The results will be entered into the eCRF. The samples will not need to be stored or sent to the central site. See Table 1 (Study Schedule).

6.4 Special Blood Tests

Blood samples will be taken at four time points (baseline, 6 months, 12 months and 18 months). 12 mL of whole blood will be collected in EDTA tubes for isolation of DNA that will be screened for single nucleotide polymorphism (SNP) for loci related to IBD. An additional 27 mL of whole blood will also be collected in SST tubes and centrifuged to obtain serum for screening of microbial antigen antibodies.[37] Assessment will be made for the relationship between changes in the microbiota or the immune system associated with recurrent disease and known genetic factors predisposing to Crohn's disease.

Whole blood samples will be at stored at –80ºC and processed serum samples will be stored at -20 ºC for future analyses as specified above.

These samples will be collected and stored locally, and then transported to the study coordinators St Vincent's Hospital in Melbourne. See section 7 for details.

6.5 Laboratory Studies

6.5.1 Factors Associated With Disease Recurrence

A prospective endoscopic study, starting at the time of absent mucosal disease, coupled with comprehensive molecular microbiological techniques, provides a unique opportunity to identify the microbiological changes associated with disease recurrence.

Endoscopically-identifiable recurrence occurs in a majority of patients within 12 months, making the identification of changes in mucosal microbiota within patients, and differences between patients with and without recurrence, relatively rapid.

Tissue and blood analysis will be performed on patients studied in the active arm of this study at two hospitals: St Vincent's and Royal Melbourne Hospitals.

For the laboratory tissue and serum studies there will be further controls ("laboratory controls"). Tissue, blood and stool will be obtained from patients without IBD having colonoscopy for family history of colorectal cancer ("healthy tissue controls"), patients with previous right colonic resection for cancer ("healthy tissue controls with resection"), and patients with ulcerative colitis ("disease tissue controls").

At surgery, mucosa will be obtained from the diseased segment and the proximal and distal resection margins.

At follow-up colonoscopy 6 and 18 months post-operatively, six to eight 1-2mm diameter biopsies will be taken from the following sites: 2-3 cm above the anastomosis, the anastomosis itself, 2-3cm below the anastomosis and the rectum. Five biopsies will be taken from diseased and non-diseased sites and stored using RNA*later*® to preserve the samples. The remaining biopsy will be stored anaerobically for specialized analysis of the anaerobic microbiota. A further two biopsies (or more, at the treating clinician's discretion) will be preserved in formalin and submitted to routine histopathology.

Blood samples are to be obtained at initial surgery, and 6 and 18 months post-operatively for: routine haematological, biochemical and inflammatory marker parameters, and microbial antigen antibodies ASCA, E. coli (Omp-C), Pseudomonas (I2) and flagellin (cBIR). [37]

Faecal specimens will be collected from the patients in the study prior to surgery and each follow-up colonoscopy. Stools will be assayed for faecal calprotectin and assessed for microbiota.

6.5.2 Microbiota Analysis

Metagenomic Analysis will be undertaken using a high throughout phylogenetic microarray together with pyrosequencing, and quantitative real time PCR assays where differences identified. ***Both the Murdoch Institute and CSIRO have extensive experience in these techniques.***[29,31,38]

(a) ***Microarray analysis.*** Extracted nucleic acid will be used in custom oligonucleotide array analysis. The microarray consists of 2400 probes, representing the known taxonomic groups in the human gut. It is derived from the GoArray program using 16S r RNA gene sequences from gut microorganisms deposited in Gen Bank.[39] Four replicates of each probe are distributed across the array.

16S rRNA gene amplification and hybridization. 16S rRNA genes from individual samples will be amplified from DNA extracts using bacteria-specific 16S rDNA primers.

Signal detection and data analysis. Images from microarray slides are scanned (Axon Genepix scanner) and signal intensities compared using GenePix software (Molecular Devices). Analysis is performed using GeneSpring 7.3 (Agilent Technologies).

Statistical analysis. One-Way ANOVA with Benjamini and Hochberg multiple test correction is performed on the log intensity ratios produced using first normalization strategy, to identify probes increased or decreased in intensity relative to a control.

(b) *Pyrosequencing analysis.* **Deep phylogenetic sequencing based on bacterial 16S rRNA Tag pyrosequencing will be undertaken using the Roche GS FLX sequencing platform.**

We will generate pyrosequencing reads from the V6 (79bp) and V3 (177bp) hyper variable regions of the bacterial ribosomal 16s gene. DNA will be amplified by PCR. At least 200,000 sequence reads will be generated and analysed for each sample at each variable region. Sequence libraries will form the basis of phylogenetic analyses.

Analysis of microbial sequences will be conducted using a suite of phylogenetic programs. A PHYLIP DNA distance matrix will be used to generate operational taxonomic units (OTU) using the DOTUR [40] and the SONS computer programs. Consensus sequences of each OTU will be used for Phylogenetic analyses using the DNA software tool MEGA version 3.1[41] .

6.5.3 Genotyping of Patients for IBD susceptibility loci:

12mls of blood in EDTA will be taken to store DNA for SNPs loci related to IBD at each major study visit.

6.5.4 Tissue, Blood and Stool Storage

Tissue from biopsies and resection specimens, blood, and stool will be stored. This relates to the possible discovery in the future of genes or pathogens that cause Crohn's disease, and our ability to test samples for these newly discovered entities.

7. STUDY VISITS

7.1 Baseline Visit

This visit should take place from 0 to 30 days prior to the surgery that leads to study entry, except where a patient is recruited post-operatively into the POCER study.

An e-CRF record must be generated for all patients who have signed the consent form for the study, regardless of post operative eligibility and/or continuing participation. .

7.2 Screening

Patients will be screened to ensure they meet all the inclusion criteria and none of the non-inclusion criteria on the day of the study baseline visit.

7.3 Inclusion

Patients will be screened at the baseline visit as above, and included only if they continue to meet the eligibility requirements following the surgical resection. Final eligibility for the study can only occur following the surgical resection.

7.4 Informed consent

Details about how informed consent will be obtained and documented are provided in the patient information and consent form. There are two forms; one for the clinical study and one for the laboratory study controls.

7.5 Medical/Surgical history and physical examination

Age (year of birth), sex, smoking status, duration of disease, age at diagnosis, height, weight, creatinine clearance, and medical and surgical history (previous resection(s), disease location, disease behaviour, medication history, current medications, CRP at time of surgery, type of surgery (laparoscopic versus open), type of anastomosis and CDAI in week prior to resection/ colonoscopy will be recorded on the day of inclusion on the CRF.

Location of patient's Crohn's disease (upper tract, small bowel, colon/rectum, and anus) and duration of the disease will be recorded on the CRF.

7.6 Post-Operative Complications

Post-operative complications occurring within the initial surgical admission must be recorded on the surgical resection form within the eCRF and as an adverse event. Any subsequent readmissions associated with the patients Crohn's disease or post-operative complications must be documented as a serious adverse event and the site Human Research Ethics Committee must be informed. It must also be documented as an Unscheduled Visit with the eCRF.

7.7 Prescription of Investigations

The investigator will prescribe all the investigations needed by the patients (these investigations will depend on whether the patient is randomized to active or standard care arms which will be determined by computerized randomization).

Following provision of information and completion of appropriate consent forms, patients will be enrolled into the study.

Clinical assessment and history of previous investigations, hospital admissions and management will be documented at the baseline visit and a booking for follow-up colonoscopy for six months (active arm) and eighteen months (active and standard care arm).

7.8 Scientific Data collection procedures

7.8.1 The Surgical Resection and Tissue Sampling

Transversely taken sections will be examined from 3 different parts of the resected bowel specimen: (1) from the diseased section, and from the macroscopically-normal (2) ileal/proximal and (3) colonic/distal margins flanking the diseased section.

The resection specimen will be sent directly to the pathologist in formalin for analysis following collection of the tissue biopsies by the study team. 16 small (0.2 x 0.2mm) samples of mucosa will be taken from each region of the bowel. 15 of these will be stored individually in 5-10 x volume of RNA*later*®, so that the solution can quickly perfuse the tissue. The samples will not be frozen immediately, but stored at 4ºC overnight to allow full penetration of the tissue. Samples will subsequently be stored at -80ºC The remaining biopsy from each region will be stored in specialised anaerobic media with Titanium III reductant, and will be immediately frozen at -80C. Samples will be couriered on dry ice to CSIRO and Murdoch

7.8.2 Follow-up Colonoscopy and Tissue Sampling

During follow-up colonoscopy at 6 months and/or at eighteen months post-operatively, eight biopsies each 1-2mm in diameter, will be taken for diagnostic processing from separate sites

within the gastrointestinal tract. This constitutes the same number of biopsies that are taken from patients with colitis as per standard government regulations as detailed in the American and British guidelines[42-44]. The sites will include 2-3cm above the anastomosis, at the anastomosis, 2-3cm below the anastomosis, and from the rectum. The biopsies may include tissue from inflamed and non-inflamed sites (i.e. from affected and unaffected sites in the same region of the gut). One of these biopsies will be sent "in formalin" for histopathology as per standard of care and one will be stored in specialised anaerobic media with Titanium III reductant, and will be immediately frozen at -80C. The remaining six samples will be stored individually in 5-10 x volume of RNA*later*®, so that the solution can quickly perfuse the tissue. The samples will not be frozen immediately, but stored at 4ºC overnight to allow full penetration of the tissue. Samples will subsequently be stored at -80ºC. Samples will be couriered on dry ice to CSIRO and the Murdoch Institute

For the colonoscopy there will be a per-rectal approach: Bowel preparation will be the same as for standard colonoscopy procedures in each hospital, with fluid only diet on the day before the procedure, and standard bowel preparation during the evening before the procedure (Colon Lytely or alternative) and an overnight fast. No phosphate-containing bowel preparation will be used.

The degree of Crohn's inflammation affecting the pre-anastomotic ileum, anastomosis, and large bowel will be recorded. The extent of inflammation at the anastomosis will be recorded, according to the Rutgeerts score, the Crohn's Disease Endoscopic Index of Severity (CDEIS), the Simple Endoscopic Score (SES) and our new POCER score (see appendices). The pre-anastomotic ileum, anastomosis, and large bowel will be photographed, and the photographs labeled as to patient name, anatomical site, and date, then kept as hard copies (and digitally entered onto CRF if possible). They will be assessed centrally in a blinded manner as part of the study analysis.

Sedation requirements are the same as for standard endoscopy procedures using conscious sedation administered by a specialist anaesthetist.

7.9 Follow-up

All patients will be reviewed at 1, 2, 4, 6, 7, 8, 10, 12, 15, and 18 months post-operatively. Patients will be reviewed by phone at two weeks post-operatively to confirm tolerance of, and compliance with the study medications. Some may be randomised to have a colonoscopy at 6 months post-operatively. All patients will have a colonoscopy at 18 months post-op. Additional visits, based on clinical need, will be available with the investigator and will be reported as unscheduled visits in the eCRF. Clinical, quality of life and healthcare utilization data collected at the baseline visit will be repeated at the six month, twelve month and eighteen month visit.

All study visits must occur within fourteen days either side of the scheduled date.

Visits at 2, 4, 7, 8, 10 and 15 months may occur by phone, provided the patient requires no intervention.

The site is responsible for ensuring that should a phone visit occur the patient still undergoes routine pathology as outlined in the study protocol.

7.10 Missed Visits

Patients must attend baseline, surgical resection, 6, 12 and 18 month visits. Non attendance at any of these scheduled visits may require the patient to drop out of the study. All other visits are important, and must be followed up rigorously by site co-coordinators. Should the patient fail to attend the other scheduled visits as outlined above, please notify the principal investigators. Routine pathology as detailed above must be performed and recorded on the eCRF regardless of a patient attendance at study visits.

8. BLOOD AND STOOL SPECIMEN COLLECTION AND STORAGE

Routine blood testing will occur at every study visit (except the surgical resection visit). Blood will be collected and tested at each study centre for: FBE, urea and electrolytes, LFTs, CRP and ESR.

Special study-specific blood and stool samples will be collected, processed, and stored at all study centres and then subsequently transported to St Vincent's Hospital in Melbourne. These will be collected at baseline, 6 months, 12 months and 18 months. A total of 39 mL will be collected by venepuncture per visit – 3 x SST tubes (9mL/tube) and 3 x EDTA tubes (4mL/tube). At each study centre, the SST tubes containing blood will be centrifuged (2000rpm for 10min) and the serum aliquoted immediately, then stored at -20ºC. EDTA tubes will be stored at -80ºC. These samples will be used for microbial antigen testing and SNP analysis (genetic testing).

Stool will be collected at baseline, 6 months, 12 months and 18 months in a stool sample container. The patient will be required to keep the stool at -20ºC in their home freezer and subsequently transport the sample on ice to the study centre, where it will be stored at -80ºCminus.

The additional blood and stool samples collected at baseline, 6 months, 12 months and 18 months will be stored at the study centre, and then transported at regular intervals to St Vincent's Hospital (Melbourne). These samples will eventually be tested for inflammatory markers (including calprotectin) and microbiology. Blood will be kept for immunological and genetic testing.

9. SAFETY DATA AND ADVERSE EVENTS

Medication/procedure related adverse events will be monitored during the procedure and at each subsequent visit, with details of the event recorded on the eCRF.

Serious adverse events will also be reported to the appropriate participating institution's Human Ethics Committee in the required format, and must be reported to the study principal investigators within 24 hours.

In the case of complications or adverse events, the patients will have full access to medical assistance through their participating institution and the research staff as named in this protocol.

A data safety monitoring board comprised of the central principal investigators and POCER study staff will meet weekly to review trial progress, address adverse events and to monitor the safety of patients.

10. STUDY DURATION

The study began in August 2009. Inclusions period will be 2-3 years from August 2009. Primary end-point at eighteen months. Extended follow-up for 5 years may occur.

11 DATA ANALYSIS

11.1 Statistical methods

Categorical variables will be applied to chi-squared tests (or Fisher's Exact tests for small samples) while continuous variables will be applied to (parametric) t-tests and (non-parametric) Mann-Whitney/Kruskal-Wallis tests for symmetrically and asymmetrically distributed data, respectively.

Confounding and allocation bias will be minimised through randomization. Information bias will be minimised by blinding the assessment of the colonoscopies by the central principal investigators. Furthermore, strict and objective criteria will be used for outcome ascertainment (that is, to define the presence or otherwise of recurrence.)

Potential selection bias may arise from the fact that subjects assigned to the standard care group may undergo escalation of management based on symptoms, including having endoscopy. This will be addressed through analysis of data based on intention-to-treat principles.

11.2 Health economic analysis

Cost-effectiveness of endoscopically guided treatment to reduce post-operative recurrence, compared to standard management, will be assessed by measuring health outcomes and health care utilization in active and control arms.

Health economic modeling will estimate potential cost-effectiveness. Decision analysis and implementation of research findings will be used to compare the downstream consequences of adoption of the new treatment algorithm versus standard care. Markov[45] and life-tabling [46]techniques will allow modeling of outcomes beyond two years.

Incremental cost-effectiveness ratios in terms of net costs per unit of health gain will be assessed. Net costs will comprise the costs of adopting the new treatment algorithm, minus costs saved from the reduction in downstream health services utilization. We will also estimate years of life and quality-adjusted life years (QALYs) gained. Both are enabled by the collection of time-to-outcome data.

To account for any uncertainty in the data inputs for health economic modeling, sensitivity and uncertainty analyses will be undertaken via Monte Carlo simulation.[47]

12. HANDLING OF PATIENT RECORDS

12.1 Source documents and case report form

Source documents are defined as original documents, data and records. This may include hospital records, recorded data from automated instruments, photographic negatives, microfilm or magnetic media.

The electronic CRF (eCRF) will be specifically designed for this study and is the data collection instrument for the study. All data requested on the CRF must be recorded. All missing data should be explained.

The eCRF will be anonymous: patient will be identified by country, study centre and their initials.

12.2 Data management

The investigator will send the data generated during the study via a secured route. Only the data recorded on the eCRF will be sent to the structure in charge of the data management.

Data will be integrated into the clinical database under the responsibility of the biostatistician.

Regular reviews of the data will be performed by the biostatistician during the course of the study and queries will be generated and submitted to the investigator for resolution if necessary.

Access to the database will be limited using passwords and regular back-ups will be performed.

13. DISSEMINATION OF RESULTS

The principal investigators will be jointly responsible for the dissemination of results arising from this project. Results will be disseminated by presentation at medical conferences and publications in peer-reviewed medical journals.

14. LEGAL AND GENERAL CONSIDERATIONS

14.1 Ethical conduct of study

The study will be conducted in accordance with the protocol, International Conference on Harmonization (ICH) guide-lines, applicable national and local requirements, and the ethical principles that have their origin in the Declaration of Helsinki.

14.2 Patient information and informed consent form

As this study will be a multi-centre randomized control trial, submission of the protocol to an independent ethics committee within each participating study centre for formal approval of study conduct is mandatory and dependent on local requirements. In addition as this study will involve intervention into patients' usual medical management, written information delivery and obtaining of patients consent is also dependent on local and national requirements.

Patient's written information and informed consent forms will be handed to each patient by the site investigator prior to inclusion in the study. The informed consent form will have to be signed and dated by the patients and collected by the investigators prior to study initiation (no procedure related to the protocol will start without the patients signed consent form). An English version of the written information and consent form is attached.

The investigators will be responsible for the storage of all the legal and or ethical documents required by the study (Ethics approval, copies of written consent form).

All the data will be rendered anonymous before being analysed.

14.3 Modification of the protocol and stopping rules

14.3.1 Modification of the protocol

Without a common agreement between all principal investigators, no alteration or modification of this protocol will be considered valid.

In case of such an agreement, the planned modifications will be subjected to an amendment with the protocol. If necessary this amendment will be submitted to the ethics committee.

14.3.2 Interruption of the study

The Principal investigators may terminate this study prematurely either in its entirety or at individual study centres for reasonable causes (e.g. unsatisfactory enrolment with respect to quantity or quality, inaccurate or incomplete data collection, falsification of records, failure to adhere to protocol). In such a case, a written notice of the intended termination will be sent to the investigator.

The investigator may also terminate the study prematurely at his/her study centre for reasonable cause, after providing a written notice to the principal investigators.

14.4 Teams involved in the project

St Vincent's Hospital (Melbourne) has one of Australia's largest inflammatory bowel disease patient cohorts, and skilled, research-supportive IBD gastroenterologists and surgeons. The study will also involve IBD-Melbourne, a collaborative clinical research group embracing 12 academic gastroenterologists around Melbourne, as well as a number of IBD research capable centres around Australia and New Zealand.

Expert molecular microbiological collaboration has been agreed with the Murdoch Institute at the Royal Children's Hospital and CSIRO, and immunological collaboration with CSL.

This study will be conducted across two countries in 21 participating gastroenterology centres (see Appendix 14 List of study centres).

14.5 Data confidentiality, access and archiving

All the information concerning the patients will be rendered anonymous and covered by the medical confidentiality.

The data collected during the study (e.g. medical measurements and demographic information) will be subjected to computerization and statistical analysis. Access rights, as provided by the law available in each participating state, can be exerted at any time by all the participating patients.

The investigator will have to store the data concerning the study for at least 15 years.

14.6 Funding source

Application currently made to National Health and Medical Research Council for a project grant.

Funding of the anti-TNF antibody adalimumab and the logistics for the cold chain supply of the drug has been granted by Abbott Pharmaceuticals.

14.7 Publication policy

Data derived from this study will be the exclusive property of the principal investigators. No use and no transmission to a third party will be made possible without its prior consent. Any publication or presentation related to the study will therefore be approved by the principal investigators. Clinical samples derived from this study may not be used by site investigators for research unrelated to this protocol, without the prior approval of the principal investigators.

The principal investigator at each clinical study site will be an author on the main clinical publication if 10 or more patients are entered into the study from that centre. Centres that enroll more than 5 patients may have an author included on a discretionary basis, as judged by the senior principal investigator at the conclusion of the study.

Authorship for each publication will be determined by the principal investigator of the study, in agreement with all participants, according to contribution made to the study, and in line with international journal standards.

15. APPENDICES

15.1 Principal Investigators Contact Details

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15. APPENDICES (continued)

15.1 Principal Investigators Contact Details (continued)

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15. APPENDICES (continued)

15.1 Principal Investigators Contact Details (continued)

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15. APPENDICES (continued)

15.2 Participating Centres

St Vincent's Hospital
St Vincent's and Mercy Private Hospital
The Royal Melbourne Hospital
Melbourne Private Hospital
The Alfred Hospital
Cabrini Medical Centre
Box Hill Hospital
Monash Medical Centre
The Western Hospital, Melbourne
Christchurch Hospital
Fremantle Hospital
Royal Brisbane and Women's Hospital
Mater Health Services Adult Hospital, Brisbane
The Canberra Hospital
The Royal Adelaide Hospital
Flinders Medical Centre
Royal Prince Alfred Hospital, Sydney
Concord Repatriation General Hospital, Sydney
St George Hospital, Sydney
Bankstown Hospital, Sydney
Liverpool Hospital, Sydney

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APPENDIX 3

Ethics Documentation, Variations and Approvals

Initial Clinical Study

Research Governance Unit
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Monday, 3 August 2009

Prof M Kamm
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SVH

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Telephone
www.svhm.org.au

Dear Prof Kamm

Protocol No: HREC-A 077/09

'Post-operative Crohn's disease recurrence: endoscopic guided intervention and determination of cause.'

The Professional Secretariat of Human Research Ethics Committee-A (HREC-A) has agreed that your latest correspondence dated Wednesday 29 July 2009, has satisfied the conditions imposed and granted full approval for this project to be undertaken at the following site/s:

St Vincent's Hospital (Melbourne)
St Vincent's and Mercy Private Hospital - Fitzroy Campus

HREC-A is constituted and operates in accordance with the NHMRC National Statement on Ethical Conduct in Human Research (2007).

HREC-A has a policy of granting approval for four years. Ethical approval is valid for four years from the date of this letter. Approval may be renewed at the end of this period by resubmission to HREC-A.

Approval is subject to:

1. Registration of the project on a clinical trial register before the first patient is enrolled (only applies if the project is a clinical trial requiring registration).

2. immediate notification to HREC-A and sponsor of any serious adverse effects on participants;

3. immediate notification of any unforeseen events that may affect the continuing ethical acceptability of the project;

4. notification and reasons for ceasing the project prior to its expected date of completion;

5. the completion of an annual report on progress of the project;

6. HREC-A approval of any proposed modification to the project; and

7. the submission of a final report and papers published on completion of project.

Please contact the department/s providing support listed below in time to allow them to prepare for the start of the trial:

Pathology
Pharmacy

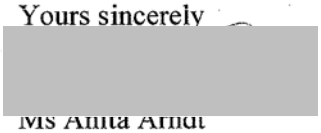
HREC approval includes the following :

Worksheet - IBDQ, final version, dated 8 August 2007

Questionnaire - Your health and well being SF-36v2

If you are using participant information and consent forms, please remember to include the HREC protocol # at the top of page 1.

Yours sincerely



Ms Anita Arndt
Senior Administrative Officer and HREC-A Secretary
Direct Tel: 9288 3924

cc: Pharmacy cc: Pathology

Amendment Clinical Study

Research Governance Unit
Ph: [redacted]

Tuesday, 3 January 2012

Prof M Kamm
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St.Vincent's Hospital
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Dear Prof Kamm

Protocol No: HREC-A 077/09

'Post-operative Crohn's disease recurrence: endoscopic guided intervention and determination of cause.'

Prof M Kamm	**Dr P De Cruz**	**Dr D Liew**	**Mr R Woods**
Dr M Johnston	**Mr R Brouwer**	**Mr J Keck**	**Mr A Heriot**
A/Prof P Desmond	**Dr W Connell**	**Dr S Bell**	**Dr R Elliott**
Dr S Brown	**Dr M Congiu**	**Dr R Norris**	**Dr Prideaux**
Ms Hamilton	**Ms Ritchie**		

The Human Research Ethics Committee-A (HREC-A) has granted final approval to the following amendment:

> Various protocol amendments to the POCER Study Protocol and associated administrative amendments (Clinical Protocol now version 2 dated 19/12/2011).

This approval will be noted by the full Human Research Ethics Committee - A at its next meeting on Wednesday 01 February 2012.

There will be no further correspondence regarding this amendment unless a member of the HREC-A raises a concern at the next HREC-A meeting.

The conditions of approval of this amendment are the same as those governing approval of the original protocol.

Yours sincerely

[redacted]

Ms Anita Arndt
Senior Administrative Officer and HREC-A Secretary
Direct Tel: 9288 3924

St Vincent's

Continuing the Mission of the Sisters of Charity

SV44812

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2 July 2015

Dr Megan Robertson
Director Research and Devlopment
HREC-A
Research Governance Unit
St Vincent's Hospital
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Dear Megan

Regarding HREC-A 077-09 – The POCER Study

The POCER Study is a completed multi-centre clinical trial, which was closed out by HREC-A on 14 October 2014. The study was closed out as it had met the primary clinical endpoint and has been published.[1]

The scientific and laboratory work on data collected during this project continues as approved. However, as this amended protocol was approved in 3/1/2012, the technology available for analysis of these samples (in line with the approved aims and hypotheses) has improved. This allows us to apply new techniques and scientific methodology to our samples and data collected during the POCER study.

We wish to ask for chairman's action to approve the following variations to the analysis of the scientific collected data. These variations will not require any additional patient contact or collection of additional samples or data, and all projects listed here comply with the initial project hypotheses and aims.

All projects listed will be subject to agreed research collaboration agreements covering project costs, intellectual property, data transfer/ownership and confidentially. These agreements will be provided to the Research Directorate as a matter of priority, prior to project commencement.

Protocol Version 2.0, dated 3/01/2012

Microbiome Analysis
Page 11
1.2 The Cause of Crohn's Disease and its Recurrence
(2) Pyrosequencing:

We now wish to use a newer more efficient technique for sequencing of the 16S gene, allowing for more detailed characterisation of the microbial community.

The initial Pilot Microbiology study referred to on page 19, paragraph 1, was completed on a limited number of patients due to limitations in the microarray and pyrosequencing techniques.[2]

We would now like to complete the remaining analyses using 16S ribosomal Illumina sequencing (performed by Murdoch Children's Research Institute collaborator Dr. Carl Kirkwood). This new, high throughput technique provides finer resolution of microbial community profile, and allows the data to be processed through newer more efficient statistical analysis pipelines. Pyrosequencing is now no longer the standard technique for microbiome analysis.

Analysis will proceed as per the hypotheses and aims and objectives listed on page 11 of the protocol, only the analysis techniques require variation.

This analysis technique will also be applied to the wider faecal sample analysis as described on pages 20 and 21 of the protocol.

We also seek to include additional targeted experiments to further investigate signals identified in the preliminary microbiology work, which may point to the influence of *Proteus spp*. in the development of post-operative Crohn's disease. Previous analysis (Wright et al, 2015, *submitted*) of the sequencing of intestinal biopsies from the POCER cohort has shown a strong preliminary link between the presence of *Proteus spp.* and the presence of endoscopic recurrence at 6 months (OR 13 (95% CI 1.1-150), P=0.039), when corrected for smoking. *Proteus spp.* are known opportunistic pathogens associated with hospital-acquired catheter infections, and possess many virulence factors that may assist in infection/colonisation of patients with Crohn's disease.[3]

Initially, the analysis will focus on definitive species identification as per paragraph 1 of page 11, reading: "*In addition genera specific quantitative real-time PCR can be employed to measure discrete populations of microorganisms.*"

Following this, we will perform additional experiments aiming to isolate the pathogen, characterise it, culture the bacteria, and undertake further studies to define its pathogenic role. This project will be undertaken in collaboration Professor Mark Morrison, Chair, Microbial Biology and Metagenomics at The University of Queensland Diamantina Institute, a previously listed collaborator on the POCER Study. Please note the change in affiliation for Professor Morrison.

Commencement of this analysis will be subject to completion of a Research Collaboration Agreement with The University of Queensland Diamantina Institute which will be submitted to the Research Directorate.

Serologic Analysis
Pages 20- 21
6.4 Special Blood Tests and 6.5.1 Factors associated with Disease Recurrence, paragraph 7.

The panel of microbial antigen antibodies available to us to utilize on the clinical samples collected has been expanded to include not only ASCA, anti-Omp-C, anti-I2 and anti-CBir1, but also pANCA (perinuclear anti-neutrophil cytoplasmic antibodies), and the additional flagellin antibodies anti-A4-Fla-2 and anti-Fla-X. These additional antibodies provide information applicable to phenotype (pANCA) and to the microbiome (anti-A4-Fla-2 and anti-Fla-X). Further, newly developed serological markers that purport to correlate with mucosal healing will be validated and tested for their clinical utility. Analysis will be performed in reference to the hypotheses and aims listed on page 11 of the original protocol.

Furthermore, the technology now exists for us to use these serum samples to specifically assess response to treatment in patients receiving adalimumab (page 12-13, 3.2 Drug Treatment). Some patients have a loss of response to adalimumab (a biologic antibody drug), related to drug levels and metabolism or to the production of anti-adaliumumab antibodies. We now have access to testing that will allow the serum samples from patients on the drug to be tested for both the drug and the antibodies against the drug and for these data to be correlated with endoscopic and clinical outcomes.

This project falls under the following hypotheses as detailed on page 11 of the protocol:

> That anti-TNF therapy will lessen recurrent disease severity in patients resistant to, or intolerant of, standard immunosuppressive therapy.

As well as the following project aim detailed on page 11:

> To examine the benefit of anti-TNF therapy in modifying disease recurrence in patients with high risk of recurrence or patients who have failed standard immunosuppressive therapy.

We are also now able to test the stool samples of these patients to determine if there is excretion of the drug through the lumen of the bowel. All of these tests can help elucidate reasons why some patients fail this valuable drug therapy. This information will be used for research purposes only, and will not be used for clinical management.

Prometheus Laboratories, San Diego, CA, will undertake the testing for these aforementioned projects in a confidential blinded fashion.

The Research collaboration agreement for this project is attached.

Genetic Analysis of Post-Operative Crohn's Disease Recurrence
Page 22
6.5.3 Genotyping of Patients for IBD susceptibility loci:

We wish to provide more information on the planned analysis of the genetic background of the POCER patients. There are only limited data on the influence of genetic background of post-operative recurrence.[4, 5] Initially, we wished to perform targeted analysis of specific single nucleotide polymorphisms (SNPs) associated with post-operative recurrence as mentioned in the approved protocol (Section 6.4, Special blood tests). However, we now have an opportunity to screen for many more SNPs related to auto-immune diseases using the ImmunoChip2 array

currently under development, in collaboration with Professor Dermot McGovern from the Inflammatory Bowel and Immunobiology Research Institute at Cedars-Sinai, Los Angeles, USA.

The ImmunoChip2 array will allow us to increase the planned number of SNPs to be assessed from approximately 30 to up to 200,000.[6, 7]

Commencement of this analysis will be subject to completion of a Research Collaboration Agreement with Cedars-Sinai which will be submitted to the Research Directorate. DNA will be extracted in the laboratories of the Department of Medicine, University of Melbourne. The ImmunoChip2 processing will be done at Inflammatory Bowel and Immunobiology Research Institute at Cedars-Sinai, Los Angeles, USA, with the chips provided at no cost. All data will be de-identified prior to analysis, which will be performed by St Vincent's study staff.

Faecal Marker Analysis
Page 21
6.5.1 Factors associated with Disease Recurrence, paragraph 7:

Since the protocol amendment, we have had the opportunity to perform testing of two additional faecal markers, as suggested on page 12, point 6:

> This study employs the current best test, endoscopic surveillance, to detect and monitor mucosal disease recurrence. However, preliminary studies suggest that stool inflammatory markers, especially calprotectin, are raised in active disease.[8] We will therefore test stool prior to each endoscopy, to determine whether faecal testing is a reliable surrogate marker for endoscopically-identified inflammation.

These two additional markers, S100A12 and Lactoferrin may provide additional accuracy for prediction of post-operative recurrence. The laboratory testing was performed by Dr. Emily Wright; a listed member of the St Vincent's POCER study research team at the same time as testing for Calprotectin was undertaken. This portion of the project was done under the supervision of Associate Professor Richard Gearry, the principal investigator for the POCER Study in Christchurch, New Zealand.

Additional analyses not listed in the Protocol

(1) Bioinformatic Systems Biology Analysis

The post-operative setting is a unique model to study disease as it re-emerges. Our completed clinical study has generated the world's largest and most comprehensive clinical, biomarker, and tissue dataset pertaining to Crohn's disease after intestinal resection, allowing for observations on disease evolution over time. This translational work, comprising diverse clinical and laboratory indices, and their statistical integration, offers a unique and original opportunity to take forward the understanding of disease pathogenesis, risk prediction and tailored therapy.

This project falls under the following hypotheses as detailed on page 11 of the protocol:

> That specific changes in gut mucosal micro-flora at a Crohn's anastomosis cause disease recurrence.

> That early changes in immune cell function / activity reflect sensitization to microbial flora, and that this activation differs in those with recurrence from those without recurrence.

As well as the following project aim detailed on page 11:

> To prospectively characterise endoscopic, histologic, microbiological and immunologic factors that are associated with disease recurrence at the anastomosis in patients having resectional surgery for Crohn's disease.

New analysis techniques now exist for us to combine and analyse ALL data collected as part of this study as one entity. This very large, well characterised dataset provides the basis for us to undertake a systems biology, multi-level (micro level – cellular responses, microbiome, immunologic data and macro level– patient history and disease phenotype, clinical and endoscopic results) network analysis.

The bioinformatic analysis aims to integrate the longitudinal clinical and scientific data obtained in the POCER Study, by utilising bioinformatic and network analysis techniques, as previously demonstrated by Jostins et al and Knights et al.[7, 9] These techniques are useful to determine possible interactions between the genetic background of the patient, the gut microbiota and the immune system.[10] The POCER study has already generated a dataset that includes patient history, genetics, disease phenotype and surgical data, along with longitudinal data on serology, microbiology, medication use and post-operative outcomes. We will use the bioinformatic and computational biology modules of the R Statistical Package[11], to link and correlate variables within the disparate datasets on a clinical (patient) level as well as to investigate the mechanistic questions of Crohn's disease pathogenesis. This work will be conducted in collaboration with Dr. Michael Inouye, of Centre for Systems Genomics, School of BioSciences at the University of Melbourne.

(2) IgA Decoration of the intestinal microbiota in post-operative Crohn's disease

We aim to utilise the samples collected within the POCER study to identify surrogate or "indirect" microbial biomarkers associated with disease recurrence, using a newly identified technique as per the attached reference. This will allow us to assess longitudinal variations in IgA decoration of gut microbiota in CD patients after "curative" resection.
This project falls under the following hypotheses as detailed on page 11 of the protocol:

> That specific changes in gut mucosal micro-flora at a Crohn's anastomosis cause disease recurrence.

> That early changes in immune cell function / activity reflect sensitization to microbial flora, and that this activation differs in those with recurrence from those without recurrence.

As well as the following project aim detailed on page 11:

> To prospectively characterise endoscopic, histologic, microbiological and immunologic factors that are associated with disease recurrence at the anastomosis in patients having resectional surgery for Crohn's disease.

Recent investigations into the gut microbiome in IBD involve molecular sequencing and bioinformatics analysis. These techniques generate enormous bodies of data, do not investigate the microbiota at the "deepest" species or strain level and require elaborate statistical processing. As a result key findings may be obscured by these processes. IBD may be caused by single species or strains present in low abundance. Alternative approaches are needed. "Beacons" or "microbial biomarkers" may point towards key organisms that are intrinsic to the inflammatory process.

We therefore propose a series of novel microbial studies that explore the microbiome indirectly: Palm et al have described the use of flow-cytometry-based microbial cell sorting based on their decoration with IgA.[12] Faecal samples from 11 IBD patients were used, and they were able to identify bacterial strains with a greater propensity for IgA binding. Culture collections of bacteria from these same 11 IBD patients were then prepared and presumptive IgA+ and IgA- binding strains were recovered (based on 16S *rrs* gene sequence homology). Mixtures of these presumptive IgA+ or IgA- strains were then used to inoculate mice, and the IgA+ consortia were found to be more colitigenic than IgA- consortia following DSS-treatment of the mice. Mono-colonization of mice with *B. fragilis* IgA+ and IgA- strains produced similar results. The findings collectively suggest that IgA preferentially binds to those members of the gut microbiota that are pro-inflammatory.

The clinical relevance will be investigated using stool samples collected during the POCER study[1], in which repeated patient sampling and analyses can be combined with endoscopic and other clinical measures of inflammation and disease course. We propose a pilot study using stool samples from CD patients collected at baseline and as part of follow up visits. Samples from 30 patients would be examined initially, divided into groups on the basis of disease course over 18 months after "curative" resection (recurrence or remission). This proposal will assess whether IgA decoration can identify bacteria driving inflammation and recurrent disease, and IgA-binding scores (and IgA-SEQ profiles) for stool microbiota of individual patients at baseline, 6 and 12 months. Lastly we will assess IgA profiles associated to endoscopic and other measures of inflammation.

This project will be undertaken in collaboration Professor Mark Morrison, Chair, Microbial Biology and Metagenomics at The University of Queensland Diamantina Institute, a previously listed collaborator on the POCER Study. Please note the change in affiliation for Professor Morrison.

Commencement of this analysis will be subject to completion of a Research Collaboration Agreement with The University of Queensland Diamantina Institute which will be submitted to the Research Directorate.

Please contact Amy Hamilton, the POCER Research Coordinator on 03 9231 2316, or amy.hamilton@svha.org.au if you require any additional information. We would be happy to provide more comprehensive research analysis plans should this be required.

Yours sincerely,

Prof. Michael Kamm
Principal Investigator
POCER Study

References

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APPENDIX

Updated Collaborator List:

The Cause of Crohn's Disease and its Recurrence (Microbiome Analysis)

Associate Professor Carl Kirkwood*
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Serologic Analysis

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Genetic Analysis of Post-Operative Crohn's Disease Recurrence

Professor Dermot McGovern
Director, Translational Medicine
Inflammatory Bowel and Immunobiology Research Institute, Gastroenterology
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Bioinformatic Systems Biology Analysis

Dr. Michael Inouye
Centre for Systems Genomics
School of BioSciences
University of Melbourne

IgA Decoration of the intestinal microbiota in Post-Operative Crohn's disease

Professor Mark Morrison*
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(*) Collaborators previously listed in Protocol Version 2.0, 3/01/2012

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15 July 2015

Professor Michael Kamm
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St Vincent's Hospital, Melbourne

Dear Prof Kamm,

HREC-A Protocol number: HREC-A 077/09
Study Closed-Out Date: 14 October 2014

'Post-operative Crohn's disease recurrence "POCER" Study: Endoscopic guided intervention and determination of cause.'

We received your letter dated 2 July 2015 requesting the re-opening of the above study (closed-out on 14 October 2014) in order to apply new techniques and scientific methodology to samples and data collected in the study. These variations will not require any additional patient contact or collection of additional samples or data, and all projects listed will comply with the initial project hypotheses and aims.

Your request has been reviewed and approved by St Vincent's Human Research Ethics Committee Chairman.

The HREC wishes you and your colleagues every success in your research.

Yours sincerely,

Azeezat Olusola Soaga
Senior Administrative Officer and HREC Secretary
Research Governance Unit
St Vincent's Hospital (Melbourne)