Factors associated with the post-operative recurrence of Crohn's disease

by

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Abstract

Background- Crohn's disease (CD) is a type of inflammatory bowel disease that can cause inflammation throughout the alimentary tract. Many individuals diagnosed with CD ultimately require intestinal resection. Unfortunately, disease often recurs following surgery. The pathophysiology of the post-operative recurrence of CD is incompletely understood, but thought to be multifactorial, involving environmental, genetic, microbial, and immunologic factors.

Aims- To identify genetic, microbial, or immunologic factors in humans that predict recurrence of CD following intestinal resection, and to modify inflammation following ileocecal resection (ICR) in a murine model using a prebiotic fiber.

Methods- Three distinct cohorts of human subjects were analyzed to determine the effect of genetic loci (n=191), microbial populations (n=45), and locoregional immunologic factors (n=26) in recurrent disease following intestinal resection. An interleukin (IL)-10 knockout murine model of ICR was utilized to assess the effect of fructooligosaccaride on post-ICR inflammation and microbial composition.

Results- A single nucleotide polymorphism associated with *BACH2* (rs1847472) predicted recurrence in our cohort (HR-1.24 CI-1.00-1.54 $p<0.05$), as did an elevation of the cytokine CCL2 in the intestinal mucosa ($p<0.01$). A lack of IL-5 and IL-16 in the regional lymph nodes of resected specimens was associated with disease recurrence ($p<0.01$). A decreased ratio of endospore-forming bacteria at the time of surgical resection was associated with disease recurrence (OR-9.2 95% CI 1.8-47.7 $p<0.01$). Supplementation of a prebiotic fiber following ICR led to increased intestinal inflammation ($p<0.05$) and a loss of microbial diversity ($p<0.05$).

Conclusion- The identification of genetic, microbial, and immunologic risk factors in the post-operative recurrence of CD supports the hypothesis that the process is multifactorial. Given the importance of BACH2, IL-16, and IL-5 in T-cell and B-cell development, adaptive immunity is

implicated in disease recurrence. The apparent importance of endospore-forming bacteria in the prevention of disease recurrence suggests a role for pre-operative microbial manipulation in the prevention of recurrent CD following intestinal resection, though the introduction of fructooligosaccharide was not successful in reducing post-ICR inflammation.

Preface

This thesis is an original work by Michael Laffin. Ethical approval for all projects was obtained from the University of Alberta Health Ethics Research Board, project names “Probiotic bacteria and epithelial cells and Breeding Colony”, AUC00000293, “The microbiology and immunology of Post-Operative Crohn’s Disease Recurrence”, Pro00028147, “Bio-bank blood & tissue GI research CEGIIR”, Pro00001994.

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The remainder of this work is original, performed by myself in consultation with my supervisory committee.

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Chapter 1- Introduction

1.1 Background

1.1.1 Overview

Inflammatory bowel disease (IBD) encompasses two relapsing systemic illnesses, Crohn's disease (CD) and ulcerative colitis (UC). Both of these diseases are characterized by chronic intestinal inflammation. This inflammation leads to abdominal pain, diarrhea, intestinal bleeding, weight loss, malabsorption, and nutritional deficiencies (Abraham, Cho and Abraham, Clara, Cho, 2009). The peak incidence of IBD is in early adulthood, typically among those aged 20 to 30 (Cosnes *et al.*, 2011). Diagnosis at this stage of life can have a significant impact on employment and social function. Up to a quarter of IBD cases are diagnosed during childhood (Benchimol *et al.*, 2009). In total, there are 230 000 Canadians living with IBD, and 10 000 new cases are diagnosed annually. In 2012, the economic cost associated with IBD in Canada was estimated to exceed $2.8 billion, and direct medical costs related to the treatment of IBD is excess of $1.2 billion. Specifically, there are 129 000 people with CD in Canada, and 5 700 new cases are diagnosed every year (CCFC, 2012).

CD is a disease characterized by transmural (i.e. full-thickness) inflammation of the alimentary tract. This stands in contrast to UC, where the inflammation is confined to the inner most layer of the bowel, called the mucosa. A more detailed comparison of these two diseases is contained in Table 1.1. The most common location for the inflammatory changes seen in CD is the terminal ileum (the final portion of the small intestine) (Thia *et al.*, 2010). Disease behaviour in CD is classically defined as stricturing, penetrating or

inflammatory (Silverberg *et al.*, 2005), and disease progression or complications from any of these disease subtypes can lead to surgery. In fact, the majority of CD patients will require surgery during their lifetime. Sixty percent of patients had an ileocolic resection (ICR) as their first surgery after the diagnosis of CD (Peyrin-Biroulet, Loftus, *et al.*, 2009). This operation involves the removal of the distal portion of the small intestine along with the most proximal portion of the colon. It is preferable to restore continuity of the alimentary tract at the time of operation by performing a surgical anastomosis. Unfortunately, within one year of the operation, disease recurs up to 80% of the time upstream of the surgical anastomosis in the neo-terminal ileum (Rutgeerts, 2003). The natural history of post-operative CD recurrence offers a novel opportunity to examine and define disease pathogenesis in its earliest stages of evolution (Buisson *et al.*, 2012). While no single causative factor has been identified, our current understanding of CD suggests that an inappropriate innate or adaptive immune response directed against normal gut bacteria is responsible for the initiation and perpetuation of intestinal inflammation (Baumgart and Sandborn, 2012). This has led to a diverse field of research on CD that examines genetics, environmental factors, and microbiology, and the impact of each on the intestinal barrier, phagocytosis, cellular function, and innate and adaptive immunity.

1.1.2 History of Crohn's disease

The first recognized description of CD was published by Morgagni in 1796 following the autopsy of a 20 year old male (Alexander, 1977):

> *"The belly, although it seemed to be not at all swelled, contained nevertheless, a great quantity of sanious ichor, which issued out of the*

intestines in many places, where they were perforated to some considerable extent. This tract comprehended the extremity of the ileum, and the nearest part of the colon besides, to the extent of two hands breadth. In that part the intestines were eroded and ulcerated, and under internal surface even affected with a gangrene, so that you see they might be easily perforated. Near to the tract some of the glands of the mesentery had grown out into a tumor, wherein was ichor, not unlike that which had burst forth into the cavity of the abdomen; but the very substance of this tumor was soft, and flaccid, and seemed to incline to corruption. The spleen was three times as large as it naturally is."

For many subsequent years, the description of the disease was sporadic and unorganized. In 1813 Combe and Saunders reported "a singular case of stricture and thickening of the ileum…the low part of the ileum, as far as the colon, was contracted for the space of three feet into the size of a turkey quill" (Combe and Saunders, 1813). Fifteen years later Abercrombie described a young female, who had thickening of her ileum with ulceration and involvement of the cecum and right colon (Abercrombie, 1828). By the middle of the 19th century, a more organized approach began to take root. Fieldings noted that from the years of 1852 to 1899 between 31 and 56 patients were seen in London teaching hospitals that had Crohn's-like disease in the small and large intestines (Fielding, 1984). In 1913, Dalziel, a surgeon from Glasgow, cited a series of nine patients in the British Medical Journal who almost certainly had CD, with involvement of the ileum, jejunum, colon, or a combination of the three (Dalziel, 1913).

The definitive work describing CD was published in 1932 in the Journal of the American Medical Association (Crohn, Ginzburg and Oppenheimer, 1932). The paper was titled “Regional ileitis” and was authored by Burril Crohn, Leon Ginzburg and Gordon Oppenheimer. They attempted to;

> *“(D)escribe, in pathologic and clinical details, a disease of the terminal ileum, affecting mainly young adults, characterized by subacute or chronic necrotizing and cicatrizing inflammation. The ulceration of the mucosa is accompanied by a disproportionate connective tissue reaction of the remaining walls of the involved intestine, a process which frequently leads to stenosis of the lumen of the intestine, associated with the formation of multiple fistulas.”*

All 14 patients in this series had undergone surgery at the Mount Sinai Hospital in New York City for a variety of clinical indications. At the initial presentation of this article surgeons from throughout the United States commented on their similar clinical experiences with CD (Fazio and Aufses, 1999).

By 1934 others reported involvement of the jejunum and colon (Fielding, 1984). This description is in keeping with the current understanding of the disease as one involving the entirety of the alimentary tract. This phenotype, while describing more extensive disease than Crohn’s original paper, was soon recognized as belonging to the same disease. In 1950 Armitage and Wilson published a paper in Gastroenterology (Armitage and Wilson, 1950) that described the reasoning behind the eponym’s popularity;

"The name Crohn's disease has been adhered to in most cases at this Hospital. It avoids confusion, makes no pretense of pathological exactitude, conveys an exact meaning, is easily remembered by students, and pays a well-deserved tribute."

In 1956 CD was thrust to the centre of attention after the President of the United States, Dwight D. Eisenhower, required an operation for obstruction (Heaton *et al.*, 1964). In retrospect he had had a lengthy clinical course, but was only diagnosed a year prior to his operation. The indication for his operation was bowel obstruction caused by a long segment of stenosis in the terminal ileum. He underwent a bypass procedure of the diseased segment.

1.1.3 History of surgery in Crohn's disease

The landmark 1932 paper, "Regional ileitis" examined 14 surgical patients, all of whom were operated on by a senior gastrointestinal surgeon, Berg, who refused authorship on the publication (Crohn, Ginzburg and Oppenheimer, 1932). His refusal, and the fact that by the time the paper was presented 12 of the 14 patients were already experiencing clinical recurrence, foreshadowed a longstanding issue with surgery in CD. More precisely, surgery does not cure CD, and recurrence occurs at a high rate in patients who require an operation. Despite the fact that medical therapies have diversified and improved, the need for surgical intervention has remained high for the CD patient (Burke *et al.*, 2013).

CD was first diagnosed by early clinicians at autopsy, but once surgical therapy on live patients was feasible, the diagnosis became a surgical one (Aufses, 2001). At first, the

disease was diagnosed by surgeons and in turn treated by surgeons. Dalziel noted that of nine patients that he treated with CD, six patients underwent successful surgical therapy and three did not have surgery (Dalziel, 1913). The described outcome of patients was poor unless they underwent an operation. Danziel and his contemporary investigators insisted that surgery was the only means of 'cure' for this disease (Fazio and Aufses, 1999). Even Crohn, himself a gastroenterologist, encouraged surgery despite the abysmal recurrence rate following surgery that was observed in his paper, "Regional ileitis" (Crohn, Ginzburg and Oppenheimer, 1932).

Initially the surgery of choice for all types of CD was either intestinal resection of the diseased segment, or a staged approach with an exclusion bypass of the diseased segment, followed by a second operation with intestinal resection. The reasoning behind the staged operation related to the decreased intestinal inflammation seen at the time of the second surgery, and the theoretically improved anastomotic healing in an abdomen without active disease (Fazio and Aufses, 1999). Debate raged between proponents of the two operations. By the 1950s the staged operation was falling out of favor (Aufses, 2001). The final impetus to abandon the staged procedure was, interestingly, due to its success in some patients. This subset of patients experienced complete disease remission with exclusion alone. Because they were clinically well, many refused a second operation, but ultimately an increased rate of adenocarcinoma of the small bowel was found in the bypassed segment. These tumours were often advanced and carried a poor prognosis (Fazio and Aufses, 1999). Intestinal resection with anastomosis remains the operation of choice in the CD patient and is further detailed in section 1.5.

1.2 Epidemiology and etiology

No single variable has been implicated as the definitive factor in the development of CD, but there are many variables that have been identified as playing a role in its development. This section will define the epidemiology of CD, and detail the role of genetics, environmental variation and microbial populations in the development of the disease, along with the interplay of these factors and their relation to the dysregulation of immune response seen in CD.

1.2.1- Epidemiology of Crohn's disease

In North America over 1.5 million people have been diagnosed with IBD, over 200 000 of which reside in Canada, and over 3 million Europeans are afflicted (Burisch and Munkholm, 2013). Worldwide incidence rates vary widely, from less than 1 per 100 000 person-years to almost 30 per 100 000 person-years (Molodecky *et al.*, 2012). CD was once seen as mainly a disease of the western world, but areas with previously low incidence and prevalence rates, including Asia, South America and southern and eastern Europe, have reported an uptick in disease (Cosnes *et al.*, 2011; Molodecky *et al.*, 2012). The incidence of CD is greatest among young adults (Cosnes *et al.*, 2011). There have been conflicting reports on whether there is a second peak of disease incidence between the ages of 60 and 70 (Cosnes *et al.*, 2011). A number of observations initially suggested that race played a role in disease development, but that evidence is largely conflicted (Kurata *et al.*, 1992; Ogunbi *et al.*, 1998; Hou, El-Serag and Thirumurthi, 2009; Abramson *et al.*, 2010). Only among the Jewish population has there been evidence of consistently elevated risk (Cosnes *et al.*, 2011). A north-south gradient has been proposed by some investigators, with a higher rate of CD in more northern geographic areas. This was

supported by a north-south gradient within the United States and Europe (Shivananda *et al.*, 1996; Sonnenberg, 2009), but this has not been the case in other regions, including Canada (Blanchard *et al.*, 2001; Nerich *et al.*, 2006). The incidence of IBD in migrants to high-prevelence areas offers interesting data, as those who immigrate at ages under 15 appear to have the highest risk of developing IBD (Probert *et al.*, 1992). Ultimately, the incidence and prevelence of CD worldwide appears to be rising (Molodecky *et al.*, 2012), but the pathology underlying these trends are poorly understood.

1.2.2- Genetics of Crohn's disease

Familial clustering in cases of IBD and CD is well recognized. A concordance study focusing on twins in northern Europe was the first large scale study to demonstrate a genetic component (Tysk *et al.*, 1988). This twin study demonstrated a 35% concordance with monozygotic pairs. On the other hand dizygotic twins were only concordant 3% of the time. Furthermore, a greater than expected level of phenotypic concordance is noted in twin studies (Halme *et al.*, 2006).

Genome wide association studies (GWAS) have identified over 140 loci that increase one's susceptibility to developing CD. A number of these loci are also shared with other associated autoimmune diseases, including type I diabetes and autoimmune thyroid disease (Burisch and Munkholm, 2013). GWAS involve the sequencing of hundreds of thousands of single-nucleotide polymorphisms (SNPs), in hundreds or thousands of individuals, and testing those SNPs for associations with disease. These studies are useful, because unlike single-gene disorders, complex diseases such as CD are caused by multiple genetic factors working together, each with a small effect. Despite the enormous scope of these studies currently genetic loci explain only 14% of CD total

disease variance (Jostins *et al.*, 2012). This may be due to the limitations of GWAS studies, including the inability to detect rare variants, structural variants, and gene-gene interactions (Manolio *et al.*, 2009). Furthermore, SNP associations are only the first step in understanding disease pathophysiology, as implicated SNPs need to be further investigated to uncover their functional implications and relevance to the disease.

The loci identified in the GWAS studies can be broadly classified into two groups, those that alter the coding portion of DNA, and those that alter non-coding DNA. Although non-coding variants make up approximately 90% of the GWAS-identified loci, they are proportionally less well-studied than those variants that are identified in coding regions (McGovern *et al.*, 2015). The mechanism of action in non-coding variants is hypothesized to be secondary to modulation of gene expression.

The functional consequences of a number of variants in coding regions have been elucidated since the discovery of their association. The assocation of a SNP within *ATG16L1* was important in defining the role of autophagy in the development of CD (Hampe *et al.*, 2007). Autophagy is a destructive cellular process involved in combating intracellular pathogens. *ATG16L1* codes for an important protein in the functioning of autophagy (Mizushima, 2003). The discovery of this association has sparked an interest in inducing autophagy in CD and other human disease (Levine, Packer and Codogno, 2015). The most well-studied genome loci is related to the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) or caspase activation recruitment domain 15 (CARD15) (Ogura *et al.*, 2001). It is the genetic loci with, at this point, the strongest link to CD (Barrett *et al.*, 2008). This gene represents an intracellular pattern recognition receptor. It is activated by a bacterial factor called muramyl dipeptide, a

component of peptidoglycan. There are three main variants associated with NOD2 and they all are hypothesized to interfere with bacterial recognition (Ogura *et al.*, 2001).

Several genetic variants seem to provide protection against the development of CD. One such gene variant is associated with *IL23R*, which confers a 3-fold protection against IBD (Duerr *et al.*, 2006). The variant produces a loss of function allele in the *IL23R* gene. This leads to numerous immunologic alterations, and suggests that IL-23 blockade may prove fruitful as a therapy for CD (Sarin, Wu and Abraham, 2011).

1.2.2.1- The special case of genetics in post-operative Crohn's disease

Little work has been done regarding the genetics of post-operative CD recurrence. To date, 3 loci associated with genes *NOD2* (Solon *et al.*, 2013)*, SMAD3* (Fowler *et al.*, 2014), and *CARD8* (Germain *et al.*, 2015) have been associated with post-operative CD recurrence. As noted, genetic variation in the gene coding for NOD2*,* an intracellular receptor for peptidoglycan (a component of bacterial cell walls) (Inohara *et al.*, 2003), is a well established risk factor for CD. Individuals who are heterozygote for the variant posses a 1.75-4-fold increase risk of disease development, whereas variants in both copies of the gene increases the risk of disease by a factor of 11 to 27 (Economou *et al.*, 2004). The *NOD2* mutation was also associated with an increased need for surgery related to CD (Cleynen *et al.*, 2013). Early studies suggested that *NOD2* risk alleles increased the risk of recurrence, but ultimately results have been equivocal (Büning *et al.*, 2004; Alvarez-Lobos *et al.*, 2005; Solon *et al.*, 2013). A variant in the gene *SMAD3* was associated with a hazard ratio of 4.04 in a single cohort of 194 patients (Germain *et al.*, 2015). *SMAD3* codes for a signalling molecule in a pathway associated with transfomring growth factor-β (Heldin, Miyazono and ten Dijke, 1997). This pathway is

involved in a variety of cellular processes including apoptosis, cell growth, cell differentiation, cell migration, and immune cell function (Massagué, 1996). Finally, homozygosity of the *CARD8* risk allele was associated with an 7.57 odds-ratio for developing recurrence of CD post-operatively (Germain *et al.*, 2015). CARD8 is a negative regulator of nuclear factor-kappa B (NF-ƙB) (Bouchier-Hayes *et al.*, 2001). NF-ƙB is prominetly involved in pro-inflammatory signaling [57]. It is important to note that these potentially crucial genetic relationships have not been replicated across multiple cohorts. Providing supporting evidence for previously identified loci, or identifying new potentially causative loci will be important in this burgeoning field of CD research.

1.2.3- Enviromental factors associated with Crohn's disease

Some have suggested that social and economic conditions in 'developed' nations might be associated with lifestyle and environmental conditions that contribute to CD. This point of view is buoyed by the fact that Asian and Hispanic peoples are experiencing an increasing incidence of CD, and that those that emigrate from low incidence regions to high incidence regions are at an increased risk (Armitage and Wilson, 1950). A plethora of factors have been posited to explain these trends to varying extents, including lower rates of breast feeding, smaller families with less crowding, improved hygiene and sanitation, availability of clean tap water, a sedentary lifestyle, exposure to air pollution, consumption of a more westernized diet, and excessive amounts of sugar and fats in easily accessible foods (Cosnes *et al.*, 2011).

The environmental exposure that is most strongly associated with CD is smoking. A 2001 meta-analysis demonstrated an odds ratio of 2 when comparing non-smokers and smokers in the development of CD (Jostins *et al.*, 2012). Further, disease severity seems

to be increased in smokers, namely in the form of elevated rates of fistula and abscess formation (Manolio *et al.*, 2009). Although there does not appear to be a dose-response relationship with the volume of cigarettes consumed, smoking cessation does lessen the frequency of clinical flares, the need for steroid medical therapy and the need for surgical intervention drops to the baseline of a CD patient who has never smoked (McGovern *et al.*, 2015). Smokers have a lower rate of response to biologics, and when they do respond, the effect does not last as long (Hampe *et al.*, 2007). Smoking is also the most recognized risk factor in regards to post-operative recurrence (Connelly and Messaris, 2014). A meta-analysis demonstrated an odds-ratio of 2.2 for recurrence in smokers (Reese *et al.*, 2008). The exact mechanism by which smoking interacts with CD is not clear, though it does not appear as if either carbon monoxide or nicotine are the cause.

A number of infective etiologies, including viruses, helminths, and specific bacteria, have also been proposed as factors in the development of CD (Mizushima, 2003). The resemblance of CD to conditions causing infectious granulomatous ileitis (e.g. tuberculosis and Johne's disease) and the fact *Mycobacterium* had been identified in the tissues and blood of CD patients led to drug trials testing antituberculous drugs. These trials have been largely unsuccessful (Mizushima, 2003).

1.2.4- Immunologic factors

The mucosal immune system's initial defense mechanism is a single layer of epithelial cells that are covered by a mucus biofilm (Darfeuille-Michaud *et al.*, 2004). This biofilm is secreted from goblet cells and is intimately associated with the bacteria present

in the gut. There is evidence that mucin creation is decreased in the CD patient due to a genetically driven decreased expression of the genes responsible for mucin, including MUC1, MUC19, and PTGER4 (Franke *et al.*, 2010). Another important element of the mucosal immune system is the preservation and management of tight junctions. These seals become leaky in CD and allow paracellular communication between epithelial cells that is not present in normal gut (Söderholm *et al.*, 2002). Further studies describe an altered adaptive inflammatory response via T-helper [Th]1 or Th17 in CD patients (Sakuraba *et al.*, 2009). This change is thought to arise from genetic defects in innate immunity, which leads to impaired clearance and increased penetration of microbes across the gut barrier. The Th cells act to control and defend against bacteria, fungi, and viruses through the release of interferon-γ, tumour necrosis factor (TNF)-α, and interleukins 17 and 22 (Barreau *et al.*, 2010). Specialized epithelial cells, called Paneth cells, also act to maintain the integrity of the mucosal immune system by producing and secreting antimicrobial granules. Paneth cells have been shown to be less effective in some CD patients (Salzman *et al.*, 2010).

The cumulative effect of these defects is increased access of luminal antigens to the lamina propria. This results in increased and inappropriate interactions between microbes and antigen-presenting cells in the lamina propria, and altered T cell response (Clayburgh *et al.*, 2005). An increasing body of evidence shows that gut microbes can guide the host's immune response while at the same time microbial populations are shaped by the presence of inflammation. As the most efficient antigen presenting cell, gut dendritic cells are at the forefront of this process (Ng *et al.*, 2011). Dendritic cells express a wide range of receptors that act to identify and interpret a variety of microbial

patterns. They then serve as a link to the innate and adaptive immune systems by expressing a broad range of innate microbial receptors (Silva, 2009). Surface Toll-like receptors (TLR)-2 and TLR-4 on dendritic cells interact with peptidoglycan and lipopolysaccharide respectively. In dendritic cells, TLR-9 functions in recognition of microbial DNA, and can elicit both inflammatory and tolerogenic responses (Madsen, 2011). The surface receptors are known to be upregulated in active CD, though little is known about TLR-9 in CD (Ng *et al.*, 2011) . Depending upon the nature of the antigen exposure, dendritic cells will release specific cytokines and express targeted co-stimulatory markers and present antigens to naïve T cells both in the lamina propria and in mesenteric lymph nodes. Intestinal dendritic cells can be defined by the $CD103^{+}$ marker in humans (Jaensson *et al.*, 2008).

The study of dendritic cells in CD provide evidence of their role in the development of pro-inflammatory T cell responses (Jaensson *et al.*, 2008; Baumgart *et al.*, 2009; Sakuraba *et al.*, 2009). Patients with CD develop higher numbers of myeloid and mature dendritic cells in the gut expressing co-stimulatory molecules CD40, CD80, CD86 and the maturity marker CD83 with an increased propensity to induce Th1 cells.

1.3 The gut microbiome

The human intestine acts as a scaffold for a multitude of microorganisms. It has been estimated that the average human's gut carries ~10^{13} microorganisms (Mai and Draganov, 2009; Sender, Fuchs and Milo, 2016). There are only a few dominant phyla found in the gut. These include Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Fusobacteria. Although these types dominate, there are over 1500

genera and thousands of different species present (Mai and Draganov, 2009). The majority of these species are anaerobic, with a density gradient that increases as distance from the acidic environment of the stomach and proximal small bowel increases. A number of factors have been demonstrated to alter the underlying gut microbiota, including diet, drugs, individual patient factors, and even temporal variation within a single patient (Vanderploeg *et al.*, 2010; Morgan *et al.*, 2012). The concept of the microbiome has been popular recently, and it has been implicated in the cause and perpetuation of multiple diseases including IBD (Eloe-Fadrosh and Rasko, 2013; Lepage *et al.*, 2013). The term microbiome was first coined in 2001 and refers to the collective microorganisms within a community (Ursell *et al.*, 2012). The rationale underlying microbiome research is that microbes, and their metabolic products, interact with their host organism in such a way that influences health. This section functions as an introduction to the microbiome, and a discussion of its role in CD.

1.3.1- The evolution of the microbiome

For over 3 billion years microbes have formed organized communities, while multicellular eukaryotic organisms have existed for only 1.2 billion years (Butterfield, Knoll and Swett, 1990; Allwood *et al.*, 2006). It is over this massive timeframe that the intertwined relationship between microbial communities and eukaryotes has evolved. This heritage is represented by the influence that microbes have on their host, which ranges from education of the immune system to alteration of energy metabolism.

Although there exists a great deal of intra-species variability, humans are more similar to one another than to other mammalian species (Ley, Hamady, *et al.*, 2008). Explanations regarding the course of evolution that resulted in the human gut microbiome

has been largely centered on two factors; 1) diet and 2) gut morphology (Ley, Lozupone, *et al.*, 2008). Humans are omnivores- that is, humans ingest both plant and meat products. Gut microbes enhance the benefit of plant product ingestion in mammals, due to microbes ability to produce simple sugars from plant polysaccharides (Russell, 2001). Consequently, classification of species as either herbivore, omnivore or carnivore reveals clustering of microbiota within these categories (Ley, Hamady, *et al.*, 2008). Furthermore, human gut microbiota has been demonstrated to be most similar to primates that are omnivores, rather than those that have a plant-oriented diet. Dietary choices have also been shown to introduce selective ecologic pressures on the microbiota in the short-term (Carmody *et al.*, 2015). Taken together, it is clear that dietary behavior influences the evolution of the gut microbiota.

Intertwined with dietary lineage is the morphology of the gut. As some mammalian species developed a solely-herbivorous niche the need for increased digestion times arose to allow for breakdown of complex polysaccharides by microbiota(Ley, Lozupone, *et al.*, 2008). Increased passage time was facilitated by enlargement of the gut. This occurred in two ways- the gut was enlarged either upstream or downstream of the stomach. These two evolutionary groups are broadly classified as foregut or hindgut fermenters respectively, and show distinct microbial profiles. This dichotomy demonstrates the impact that alimentary morphology can have in the evolution of gut microbiota.

Ultimately, the evolution of the gut microbiota has left humans with a distinct pattern of microbial species that interacts with the human host throughout the host's life.

1.3.2- The microbiome changes through life

The interaction between an individual and its microbiome begins at birth, and in humans the method of delivery defines the key players. This is a critical timeframe to understand, as ecologic theory posits that the initial species define the environment in a unique way that leads to the fully realized adult ecosystems (Connell and Slatyer, 1977). A vaginal birth leads to the newborn's microbiota being dominated by those microbes commonly found in the mother's vaginal canal- namely *Lactobacillus* and *Prevotella* species (Dominguez-Bello *et al.*, 2010). This stands in contrast to those born via Caesarean section who inherit microbes associated with skin (Dominguez-Bello *et al.*, 2010). Maternal breast milk plays an important role in shaping the early development of the microbiota by promoting the fitness of certain microbes, such as *Bifidobacterium*, over other species (Marcobal *et al.*, 2010). The presence of facultative anaerobes in the gut at this point is crucial, as they reduce the environment and allow the strict anaerobes that form the basis of the mature microbiotas to take hold (Favier *et al.*, 2002). This allows a rapid increase in microbial diversity in the first years of life (Palmer *et al.*, 2007). As the individual reaches maturity, the microbiota becomes more stable, though the influence of changes in lifestyle, wellness, and sexual maturity continue to produce alterations in adulthood (Dominguez-Bello *et al.*, 2011).

1.3.3- The gut microbiome, immunity, and disease

There is a complex interplay between the microbiota and the development of immunity, which is evidenced in a number of ways. First, animals raised in a germ-free environment develop a different immune structure than their conventionally raised counterparts, demonstrating smaller Peyer's patches (Abrams, Bauer and Sprinz, 1962), reduced

CD4+T cells (Helgeland *et al.*, 1997), and other changes (Smith, McCoy and Macpherson, 2007). Next, the difference in microbiota between vaginally birthed humans and those born via Caesarean section has been associated with early-life skin infections (Penders *et al.*, 2007), and development of atopic diseases, including allergy, and asthma (Bager, Wohlfahrt and Westergaard, 2008). Naturally occurring commensal bacteria are important in establishing regulatory responses in the gut. For example, signals from certain bacteria help establish tolerance (i.e. beneficial immunosuppression to orally ingested antigens) that would otherwise lead to inappropriate intestinal inflammation (Weiner *et al.*, 2011). Finally, bacterial metabolites seem to play a role in in controlling various aspects of the immune system. The main group of metabolites studied in this regard are short-chain fatty acids (SCFA), which are a product of bacterial fermentation. SCFA have an effect on gene expression (Davie, 2003), and modulate the induction of regulatory T-cells (Furusawa *et al.*, 2013).

The inverse of the relationship between microbes and immunity involves the effect of the immune system on microbial ecology and community structure. Early in life, a tolerogenic immune profile develops. This environment is characterized by an attenuated inflammatory response and shift in B and T cell populations toward a tolerant profile (PrabhuDas *et al.*, 2011). This enables a healthy microbiota to take hold, while avoiding inflammation. Next, model organisms with programmed predictable genetic defects altering immunity exhibit dysbiosis. For example, *TLR5* knockout mice, which lack an important protein for identification and response to bacterial flagellin, exhibit altered microbial composition (Vijay-Kumar *et al.*, 2010).

Given the interplay between the microbiome and immune response, it is not surprising that the microbiome has been associated with a number of diseases. This includes acute microbial infections with specific identified organisms, such as *C. difficile*, *Campylobacter*, and *Salmonella* among others. There are other, more chronic diseases that are intertwined with the gut microbiota such as type II diabetes mellitus (DMII). DMII is a disease characterized by a deficit in insulin secretion coupled with insulin resistance (ADA, 2013), and has been recognized as an inflammatory condition with increased production of systemic inflammatory cytokines (Gregor and Hotamisligil, 2011). Individuals with this disease demonstrate an increased ratio of Firmicutes compared to Bacteroidetes, and the microbiota of obese mice, when transferred to healthy mice, can lead to obesity (Turnbaugh *et al.*, 2006). The development of gastrointestinal cancers have been associated with specific microbes, most famously *Helicobacter pylori* in the development of peptic ulcers and gastric cancer (Marshall and Warren, 1984). Disease susceptibility to colon cancer in a mouse model has been shown to be transferred through the microbiota (Garrett *et al.*, 2009; Hu *et al.*, 2013). Although many animal experiments seem to point to a role for the microbiome in the development of disease, it has been difficult to untangle the cause-and-effect relationship of disease and the associated microbiota in human disease.

1.3.4 The gut microbiome in Crohn's disease

Given the interconnected nature of immunity and inflammation, it is not surprising then that when compared to patients without IBD, CD patients exhibit a "dysbiosis" of their gut microbiota. This phenomena has been well described in CD patients, but whether these changes are causative or associative remains to be seen (Ogura *et al.*, 2001;

Barrett *et al.*, 2008; Pascal *et al.*, 2017). A number of differences are described in the literature, documenting changes in the number of microbes present, alterations in community composition, increased adherence to mucosa, invasiveness or virulence of select species, and alterations in functional and metabolic characteristics (Ogura *et al.*, 2001; Barrett *et al.*, 2008). Some microbes have been associated with decreased inflammation in CD, namely *Bifidobacterium and F. prausnitzii* (Vanderploeg *et al.*, 2010).

As mentioned in section 1.2.2, recent human genetic studies also strongly implicate microbes in CD pathogenesis (Duerr *et al.*, 2006). Genome-wide association studies have linked CD to several genes involved in innate immune responses to intestinal microbes (e.g. NOD2) and clearance of intracellular microbes through autophagy (e.g. autophagy related 16-like protein 1; and immunity-related GTPase M, IRGM) (Shivananda *et al.*, 1996). Animal models that have a genetic pre-disposition to pancolitis will not develop inflammation in a germ free-environment, but will when raised in a normal bacteria-rich environment, emphasizing the role of bacteria in this inflammatory process (Sarin, Wu and Abraham, 2011). These recent discoveries clarify the microbial basis of CD, implicating defective containment, killing, and clearance of invasive or intracellular bacteria by the host in the onset of CD.

1.3.5- Tools to modulate the microbiome

Significant effort has been expended developing therapies to modulate the gut microbiome. This has proven to be challenging, given the microbial differences at baseline between individuals and consequent variable response to therapy. Probiotics are ingested microbes intended to provide health benefits. Strains tested in IBD include lactic acid bacteria, *Bifidobacterium, Saccharomyces,* and strains of *E. coli* (Martín *et al.*,

2013). The results of probiotic use in induction and maintenance of remission has been underwhelming (Lichtenstein, Avni-Biron and Ben-Bassat, 2016), though a recent study demonstrated that a proprietary probiotic mixture, VSL#3, may be helpful in reducing the severity of recurrence in CD post-operatively (Fedorak *et al.*, 2015). Genetically-engineered probiotics that produce anti-inflammatory metabolites such as IL-10, or KGF2 have had success in reducing colitis in animal models (Steidler, 2000; Hamady *et al.*, 2010).

Fecal microbiota transplant (FMT) has been employed as a therapy attempting to restore healthy microbial populations since the 4th century (Zhang *et al.*, 2012). FMT is successful in the treatment of *C. difficile* infection, with approximately 90% of patients responding (Kassam *et al.*, 2013). The purpose of FMT is to introduce normal commensal bacteria population that outcompetes virulent strains for ecologic niches in the gut. This approach decreases inflammation initially in IBD, but remission may not be sustained in those who respond to FMT (Laffin and Madsen, 2017).

Parasitic worms, by their very nature, evolved mechanisms to modulate host immunity and prevent inflammation to facilitate colonization (Whelan, Hartmann and Rausch, 2012). These worms are largely eradicated in industrialized countries (Elliott and Weinstock, 2012), but some evidence demonstrated a favorable microbiota and decreased susceptibility to colitis following parasitic infection (Walk *et al.*, 2010; Whelan, Hartmann and Rausch, 2012).

Antibiotics are effective in modulating the microbiome (Jakobsson *et al.*, 2010) and have been shown to have beneficial effects for patients with CD, but their side-effect profile is undesirable for long-term therapy (Su, Ma and Zhang, 2015). Other emerging

therapies include vaccines targeting specific microbes associated with CD (Borody, Peattie and Campbell, 2013), and introduction of selective bacteriophage therapy (Golkar, Bagasra and Gene Pace, 2014; Jin, Hongyu and Jun, 2014).

An intriguing branch of work regarding modulation of the microbiome in CD relates to prebiotic usage. Prebiotics are “a selectively fermented food ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health” (Roberfroid, 2007). The main candidates for effective pre-biotics are trans galacto-oligosaccharide, lactulose, lactosucrose, soya-oligosaccharides, xylo-oligosaccharides, arabinoxylan-oligosaccharides, inulin, and fructo-oligosaccharide (FOS) (Verspreet *et al.*, 2016). These fibers have different biochemical makeups, including differing degrees of polymerization, and are fermented in different parts of the gut, by different microbes. The only prebiotic that has shown a trend toward efficacy in randomized control trials in CD has been FOS. A 2011 trial randomized 103 patients to FOS or placebo, and those receiving FOS showed a trend toward increased clinical response (39% versus 22% respectively, p=0.067) (Benjamin *et al.*, 2011; Ghouri *et al.*, 2014).

FOS can occur naturally in plants including chicory and onion among others (Rivero-Urgell and Santamaria-Orleans, 2001). Its chemical structure consists of a variable number of fructose units (usually between 2 and 60) with a terminal glucose linked by β-(2-1) bonds (Sabater-Molina *et al.*, 2009). FOS has been shown to be bifidogenic, including in the CD population specifically (Lindsay, 2006), which has driven interest in FOS given *Bifidobacterium’s* association with decreased disease activity in CD (Vanderploeg *et al.*, 2010). FOS has demonstrated an ability to modulate the gut immune

system, as infants fed a diet enriched with FOS demonstrated increased fecal IgA (Bakker-Zierikzee *et al.*, 2006). A beneficial effect of FOS may also relate to its association with increased gut short-chain fatty acids (SCFA). SCFA may improve gut barrier function and exert anti-inflammatory effects (Tan *et al.*, 2014). Elevated dietary FOS has been shown to increase SCFA in the gut (Whelan *et al.*, 2005). Ultimately, FOS is a promising prebiotic, that may prove to be beneficial in patients with CD, or other types of inflammatory microbial dysbiosis.

1.4 Current state of Crohn's disease

CD has been recognized since 1932 (Crohn, Ginzburg and Oppenheimer, 1932), but the development of therapeutics for the disease has accelerated in the last two decades. The care of those with CD has never been more complicated, or more promising.

1.4.1- The natural history of Crohn's disease

Over 80% of CD patients are diagnosed prior to the age of 40 (Freeman, 2007). There is substantial variation in the presenting symptoms, and they typically include some combination of diarrhea, abdominal pain, and weight loss (Freeman, 2014). Although CD is defined by bowel inflammation, extra-intestinal manifestations are occasionally the only presenting symptom with intestinal inflammation confirmed afterward. Occasionally CD presents at a very advanced stage, be it extensive stricturing, inflammation, free perforation of the enteral tract, intra-abdominal abscess formation or following the development of extensive fistulae (Freeman, 2005, 2014). It is difficult to define disease onset (as opposed to time of diagnosis) in CD, because a significant lag can exist between the initiation of disease activity and diagnosis in a clinical setting (Heikenen *et al.*, 1999).

Therefore, if there is a causative event in the development of CD, it may not be obvious at the time of diagnosis. The early stages of CD are marked by granulomatous inflammation. CD progresses to the large ulcers, strictures, and fistulas that are problematic in the clinical management of the disease (Kelly and Sutherland, 1988).

The variability of the initial presentation of CD is mirrored by the variety of disease subtypes seen in individuals with CD. All patients have a chronic course, but with different key clinical features (Freeman, 2014). These features were defined at the widely cited 2005 World Congress of Gastroenterology meeting, which took place in Montreal (Silverberg *et al.*, 2005). The classification scheme defined during the meeting, known as the Montreal classification (Satsangi, 2006), is validated and widely utilized in CD literature (Spekhorst *et al.*, 2014). This system classifies an individual's disease based on the age of diagnosis, disease location, and disease behavior. Disease behavior can be classified as either stricturing, penetrating, or non- stricturing/non-penetrating, with a modifier added if perianal disease is present. The most common disease location is the terminal ileum and colon, but inflammation can be present anywhere throughout the GI tract (Freeman, 2007).

1.4.2- Medical treatment options

The initial approach to the management of CD can be broadly classified as either taking a 'step-up approach' where less potent therapies are initiated first, followed by more potent therapies should the initial treatment fail, or 'top-down approach' where the most potent therapies are started initially. A controlled trial examining these two approaches demonstrated that the 'top-down approach' resulted in increased clinical remission at 26 and 52 weeks, though clinical equipoise regarding the optimal approach

still exists given the side-effect profile of more potent therapies (D'Haens *et al.*, 2008). The medications currently used to treat CD can be classified as immunomodulators (including biologic therapies), 5-ASA drugs, antibiotics, and corticosteroids (Lichtenstein, Hanauer and Sandborn, 2009).

The newest drug class in the treatment of CD are a form of immunomodulators called biologic therapies, or 'biologics'. The most widely used biologics are anti-tumor necrosis factor (TNF) drugs, the three most prominent examples being infliximab, adalimumab, and certolizumab. All three have demonstrated efficacy in luminal CD (Peyrin-Biroulet *et al.*, 2008; Colombel *et al.*, 2010). Infliximab is a chimeric monoclonal antibody used to treat a number of autoimmune diseases, including psoriasis. It is an artificial antibody that was developed in mice and engineered for humans. Adalimumab is fully derived from human monoclonal antibodies (Brekke and Sandlie, 2003) and was developed for use in rheumatoid arthritis. It is now also used in psoriatic arthritis, ankylosing spondylitis, and IBD. Certolizumab is a humanized, PEGylated anti-TNF-alpha antibody developed for the treatment of rheumatoid arthritis and CD (Goel and Stephens, 2010). All three anti-TNF agents have been shown to be very potent in inducing and maintaining remission of CD, but due to their relatively recent discovery, long-term efficacy and side effects have not been well described. There are a number of alternative emerging biologic therapies, including anti-integrin treatments such as vedolizumab (Sandborn *et al.*, 2013). Integrin plays a role in leukocyte migration into enteric tissues, and treatment targeted against integrin has provided promising results (Reenaers, Louis and Belaiche, 2010). The anti-IL12 and IL23 antibody, ustekinumab, has also demonstrated efficacy in the treatment of CD (Feagan *et al.*, 2016).

Other immunomodulators used in CD include methotrexate and azathioprine (including its active metabolite, 6-metacaptopurine). Methotrexate and its breakdown products act to inhibit enzymes in the metabolic pathway of folic acid synthesis. At high doses, methotrexate is responsible for inhibition of DNA, RNA, and protein synthesis, while at lower doses it decreases other folate dependent enzymes that promote and propagate inflammation (Herfarth, Long and Isaacs, 2013). Its mechanism of action in CD is not well-defined (Egan and Sandborn, 1996). The data surrounding methotrexate's efficacy is mixed, though one randomized trial demonstrated a benefit in induction of remission (Feagan *et al.*, 1995; McDonald *et al.*, 2014). Azathioprine is a pro-drug that is rapidly converted to 6-mercaptopurine following ingestion (Nielsen, Vainer and Rask-Madsen, 2001). Administration of azathioprine has a potent effect on immune cells, inhibits proliferation of B and T lymphocytes, and decreases levels of plasma cells and cytotoxic T-cells (Nielsen, Vainer and Rask-Madsen, 2001). Treatment leaves the patient at risk of bone marrow suppression (McDonald *et al.*, 2014). Azathioprine is not effective in inducing remission of CD, but may play an important role in maintaining remission of disease (Terdiman *et al.*, 2013; Chande *et al.*, 2016).

Glucocorticoids, in the form of prednisone and hydrocortisone, are used in CD therapy. They may be used in acute flares, and as rescue medications when other classes of medication have failed. They act through numerous pathways to decrease inflammation (Sandborn *et al.*, 2012). The benefit in the induction of remission with the use of glucocorticoids has long been established (Summers *et al.*, 1979; Malchow *et al.*, 1984).

Antibiotics have a clear role in the treatment of CD complications, such as intestinal perforation, abscess formation, and perianal disease but their benefit in treating the inflammatory process itself is unclear (Khan *et al.*, 2011). The most well-studied antibiotics in this regard are ciprofloxacin, rifaximin, metronidazole, and clarithromycin. These have shown varying degrees of success, with more effect in CD of the colon than the ileum. Rifaximin offers the most promise given its modest side-effect profile, but its effects are not clearly established (Jakobsson *et al.*, 2010; Scribano and Prantera, 2013).

Aminosalicylates (5- aminoalicylate acid, sulfasalazine, mesalamine) have not consistently shown an effect in CD (Ford *et al.*, 2011). They have anti-inflammatory properties that are not fully elucidated but may be mediated through a number of pathways (Stevens *et al.*, 1995; Gadangi *et al.*, 1996; Haskó *et al.*, 2001). Research has offered conflicting evidence on the efficacy of this drug class, and this is echoed by the disparate recommendations by experts and gastroenterological societies (Travis, 2006; Lichtenstein, Hanauer and Sandborn, 2009; Ford *et al.*, 2011; Mowat *et al.*, 2011).

These therapies make up the mainstays in the medical management of CD, either as lone agents, or in combination with one another. Despite the litany of options available, failure of treatment is common, and the need for surgical therapy in the form of intestinal resection remains high (Canin-Endres *et al.*, 1999).

1.5 Surgery and Crohn's disease

The indications for abdominal surgery in CD can be broadly placed into two categories; failure to respond to medical management and, complications arising from the disease. CD complications that lead to surgery include stricturing, fistula formation, free

perforation, abscess, bleeding, malignancy, pre-malignancy, and growth retardation. It is important to note that peri-anal complications often lead to surgery as well, but the decision making, and management of these issues is very different from abdominal surgery and will not be addressed here.

Intestinal stricturing occurs in between 20 and 40% of CD patients (Cleynen *et al.*, 2013; Yamamoto and Watanabe, 2014). These strictures demonstrate thickening of the full-thickness of bowel wall, along with increased extracellular matrix and mesenchymal cells (Burke *et al.*, 2007; Rieder and Fiocchi, 2013). The prolonged inflammation and fibrosis associated with CD ultimately leads to stricturing, which can occur at any point in the gastrointestinal tract (Strong *et al.*, 2015). Strictures respond poorly to medical therapy, especially if the stricture is due to fibrosis rather than inflammation (Chang *et al.*, 2015). The presentation of stricturing disease is often that of obstructive symptoms, and can be very uncomfortable for the patient. These symptoms, in the face of failing non-procedural treatment, act as an indication for surgery.

A fistula is a tract of tissue between two epithelial surfaces (Sandborn *et al.*, 2003). Two-thirds of CD-related fistula form in the perianal region, while enteric fistulas- comprised mainly of entercutaneous (bowel-to-skin), enterovesicular (bowel-to-bladder) and enteroenteric (bowel-to-bowel) fistula- make up the remaining third (Schwartz *et al.*, 2002). Surgery typically follows prolonged failure of medical management, though intra-abdominal sepsis or hemorrhage often necessitate earlier operative intervention (Levy *et al.*, 1989; Gecse *et al.*, 2013).

Free perforation of the large or small bowel is a potentially life-threatening complication of CD that always necessitates prompt operative intervention (Strong *et al.*,

2015). Up to 10% of patients may present with free perforation at some point in their disease course, typical from the small bowel (Werbin *et al.*, 2003). Perforation typically is presumed to be secondary to severe ulceration in a diseased segment of bowel (Strong *et al.*, 2015). Contained perforations typically result in intra-abdominal abscess formation. This can be life-threatening, but in the era of antibiotics and radiographically-guided drainage, an abscess can typically be managed without operative intervention. If the abscess cavity is less than three centimeters it can be treated with antibiotics alone (Feagins *et al.*, 2011). If larger than three centimeters in any dimension, it typically requires antibiotics plus percutaneous radiographically guided drainage. If the abscess is large, loculated, colonic in origin, or the patient is on steroids the risk of failure with a non-operative approach is high (Da Luz Moreira *et al.*, 2009; Bermejo *et al.*, 2012).

Frank hemorrhage is an uncommon complication of CD (Robert, Sachar and Greenstein, 1991). Initial management is that of any massive GI bleed, and consists of resuscitation and attempting to localize the source. If the bleeding is halted, prompt initiation of medical therapy decreases the incidence of re-bleeds (Kim *et al.*, 2012). Intractable bleeding almost always necessitates salvage surgery (Daperno, Sostegni and Rocca, 2012).

The development of intestinal neoplasia has been associated with CD (Laffin and Karmali, 2015). Patients who have suffered from CD for a prolonged period of time should undergo endoscopic surveillance for neoplastic disease (Strong *et al.*, 2015). Small-bowel carcinoma is 19 times more like to occur in the patient with CD than the population at large (Laukoetter *et al.*, 2011). Given this association, lesions at any point in the GI tract should be evaluated critically, and if suspected to be cancerous, necessitate surgery.

In the pediatric CD population, up to 40% of children suffer from growth retardation (Walters and Griffiths, 2009). The etiology of growth failure is multifactorial, driven by local and systemic hormonal changes, along with nutritional deficiencies (Heuschkel *et al.*, 2008). These changes are seen as an impact of inflammation, and there is dramatic evidence that resection of diseased bowel reduces energy expenditure and allows catch-up growth (McLain *et al.*, 1990). Therefore, prepubertal patients experiencing growth failure despite aggressive medical therapy should undergo surgery (Strong *et al.*, 2015).

Finally, surgery is indicated in those who fail to respond to adequate medical therapy in terms of the primary inflammatory disease process. Ultimately the goal of therapy in CD is to induce remission in those with active disease, and to maintain remission in those with inactive disease. Once inflammation becomes established in the gut of a CD patient, the situation can be very challenging to treat. These cases present an uphill battle for clinicians, especially when attempting to use medical therapies alone. Should medical therapy fail to achieve these ends, for any number of reasons ranging from non-compliance, to inability to tolerate the side-effect of medications, surgically-induced remission should be considered. Surgical remission offers a relatively inflammation free environment for medical interventions in the post-operative period.

1.5.1- Operative considerations

The pre-operative management of CD patients is important to consider, given that those undergoing surgery have often failed medical therapy and can be very ill. Optimization may include the use of parenteral nutrition, and discontinuation of immunosuppressive medications (White *et al.*, 2012; Kotze and Coy, 2014). IBD patients

have been shown to be at a higher risk for the development of deep vein thrombosis, and appropriate anti-thrombotic prophylaxis is paramount (Nguyen *et al.*, 2014).

The choice of operation then depends on a number of factors, including characterization of the disease phenotype, evaluation of symptoms, underlying comorbidities, and pre-operative status (Strong *et al.*, 2015; Schlussel, Steele and Alavi, 2016). Often, in cases of sepsis and abscess formation, the initial operation is the first step in a staged approach- namely source control of the sepsis, followed by a second operation that deals with the diseased segment of bowel. The definitive operative options for CD include intestinal resection with creation of an anastomosis, intestinal resection with creation of ileostomy, and strictureplasty.

Intestinal resection with anastomosis is the most commonly performed operation in the CD patient. With modern anesthesia, fluid and electrolyte management, resection is both safe and effective in controlling the acute phase of the disease or complications secondary to the disease. The most common abdominal operation performed in CD is ICR with creation of a small bowel to large bowel anastomosis (Buisson *et al.*, 2012). Principles of intestinal resection and anastomosis in these patients mainly focus on maximizing anastomotic patency, due to the tendency for obstruction if disease recurs, and minimizing the amount of healthy bowel removed at each operation. It has been proposed that a side-to-side anastomosis results in a wider lumen and therefore is less likely to lead to stasis of feculent material, microbial perturbations, disrupted barrier function, and ultimately recurrence (De Cruz *et al.*, 2012). This is not consistently born out in the evidence, with side-to-side offering comparable outcomes to other anastomotic configurations (Simillis *et al.*, 2007; McLeod *et al.*, 2009; He *et al.*, 2014). Similarly, there

is not overwhelming evidence favoring either the creation of a hand sewn anastomosis or a stapled anastomosis (Choy *et al.*, 2007). A recently developed anastomotic configuration, the Kono-S, has shown promise in optimizing anastomotic luminal diameter, and potentially decreasing symptomatic recurrence (Fichera, Zoccali and Kono, 2012).

The extent of intestinal resection is important to consider. The surgeon must balance the desire to remove diseased tissue, while also preserving the maximum amount of bowel. Historically, wide resection margins were considered the standard, with the hope of avoiding post-operative recurrence (Bergman and Krause, 1977). However, a randomized trial demonstrated that an extended resection was no more likely to prevent recurrence than a limited resection, and that even the presence of microscopic disease in the margins of the resected specimen did not predispose one to recurrence (Fazio *et al.*, 1996). Current recommendations suggest resecting only macroscopically active diseased bowel at the time of surgery (Strong *et al.*, 2015).

Following resection, the creation of a stoma (an intentional surgically created connection between the intestinal lumen and anterior abdominal wall) is common in the treatment of CD patients (Cataldo, 2008). The most common form of stoma in these patients is ileostomy (where the ileum is brought to the skin). It is an important therapy in treating the critically ill patient, such as those who have freely perforated their bowel, or in those with extensive colonic disease. Creation of a stoma, has a significant effect on the patient's quality of life and therefore should not be undertaken without great consideration (White and Hunt, 1997; Brown and Randle, 2005). It is interesting to note that patients having undergone fecal diversion in the form of stoma creation, almost never

develop CD disease in the downstream portion of their bowel (Rutgeerts *et al.*, 1991). This speaks to the importance of the fecal stream in initiating disease activity.

In the vein of bowel preservation, strictureplasty has been advocated as an alternative to bowel resection for some cases of stricturing CD (Schlussel, Steele and Alavi, 2016). Strictureplasty involves the opening of the bowel along the length of the stricture, and closure in such a way that increases the bowel's luminal radius. The most common type of strictureplasty performed is the Heineke-Mikulicz, comprising approximately 80% of cases (Yamamoto, Fazio and Tekkis, 2007). There is a significant role for strictureplasty in the CD patient when the preservation of bowel is paramount (Hassan *et al.*, 2007; Schlussel, Steele and Alavi, 2016).

1.5.2- Post-operative recurrence

The natural history after ICR nearly always involves recurrence of CD at the surgical anastomosis. Signs of recurrence are present in the mucosa of up to 85% of patients after one year, typically in the upstream portion of bowel (Buisson *et al.*, 2012). Clinical recurrence rates approached 40% at one year. By 10 years, repeat surgery is required in over half of these patients (Rutgeerts, 2003).

There are a number of ways to define and assess post-operative recurrence, which provide different information regarding the patient's clinical course and the fundamental pathophysiology of the disease. One straightforward way to define recurrence, is by assessing the need for reoperation. It is useful when the data to be analyzed is retrospective, because the need for surgery is a clearly defined endpoint that is almost always well-documented. Follow-up must be extensive to maximize the utility of this

endpoint, as the progression to surgical intervention often takes many years. Clinical recurrence scores are commonly employed and are based upon signs and symptoms of disease. A popular scoring system is called the Crohn's disease activity index (CDAI) and takes into account factors including the presence of abdominal pain, frequency of bowel movements, and general well-being (Rutgeerts *et al.*, 1990). Clinical symptoms often show a delayed onset compared to endoscopic lesions, and therefore endoscopic recurrence scores are often the preferred outcome measure for clinicians and researchers alike. Furthermore, early endoscopic assessment and subsequent alterations in medical management, has been associated with improved disease control following intestinal resection in CD (De Cruz, Kamm, *et al.*, 2015). Endoscopic recurrence is typically characterized by aphthous and serpiginous ulcerations. The most commonly used scoring system was developed in 1990, and is called the Rutgeert's scoring system for post-operative recurrence of CD (Table 1.2) (Rutgeerts *et al.*, 1990). Other methods of defining recurrence that are gaining prominence include the measurement of serologic markers of inflammation (i.e. C-reactive protein, and erythrocyte sedimentation rate) and radiographic assessment of disease (Connelly and Messaris, 2014).

The work done to define post-operative recurrence of CD facilitates two goals; first, prompt and appropriate management of CD in the individual, and second, to serve as the reference standard in studies defining risk factors for the development of recurrence. These risk factors can be largely grouped into three groups, namely patient factors, disease behavior, and environmental factors.

Patient factors include those elements that are innate to the patient, and generally are viewed to be unmodifiable. For example, a family history of IBD has been associated

with increased risk of reoperation (Unkart *et al.*, 2008). Also, as discussed above the genetic make-up of an individual has been linked with the development of post-operative CD recurrence. Briefly, 3 loci have been suggested to have an association with post-operative CD recurrence- *NOD2 , SMAD3*, and *CARD8* (Solon *et al.*, 2013; Fowler *et al.*, 2014; Germain *et al.*, 2015). These results have not been replicated across multiple cohorts. The identified *NOD2* defects impair human cellular response to components of the bacterial cells wall and therefore act as modulators of inflammation (Nakagome *et al.*, 2012; Philpott *et al.*, 2013). *SMAD3* mediates the activity of transforming growth factor-beta, which is an important signaling molecule in the inflammatory response (Derynck and Zhang, 2003). Finally, *CARD8* interacts with NOD receptors and plays a role in the development of the inflammasome, which suggests a role in both innate and adaptive immunity (Bouchier-Hayes *et al.*, 2001; Pathan *et al.*, 2001; Razmara *et al.*, 2002; Yamamoto *et al.*, 2005). Other patient factors have not been demonstrated to consistently predispose one to recurrence, including patient sex, age at diagnosis, or age at surgery (Connelly and Messaris, 2014).

Certain disease patterns, or phenotypes, have been associated with an increased risk of recurrence following resection. Disease that affects a large portion of the gastrointestinal tract, dubbed widespread disease, is associated with increased recurrence following surgery (Heimann *et al.*, 1993; O Bernell, Lapidus and Hellers, 2000). The presence of perianal disease has also been noted as a risk factor for increased recurrence after an intra-abdominal resection (Sandborn *et al.*, 2003). Evidence regarding rates of recurrence between patients with different Montreal Classifications of disease behavior is mixed, with no clear association between recurrence and stricturing,

penetrating, or inflammatory disease being consistently noted (Connelly and Messaris, 2014). A number of studies have also noted that the presence of granulomatous disease is associated with increased recurrence risk (Rutgeerts *et al.*, 1984; Anseline, Wlodarczyk and Murugasu, 1997; Cullen *et al.*, 2007; Malireddy *et al.*, 2010).

The environmental factors that a post-operative patient is subjected to can play a role in recurrence. Collectively these are termed the “exposome”, and include macroscopic factors, such as drug treatments, and microscopic features, including luminal bacterial metabolites. Smoking is a recognized environmental risk factor for recurrence and is associated with an odds ratio of 2.2 for clinical post-operative recurrence (Yamamoto, 2005). The mechanism by which smoking exerts this negative effect is not clearly elucidated. The use of medical therapies to prevent recurrence has been inconsistent. Two trials have employed nitroimidazole antibiotics with some success, but the risk profile of prolonged antibiotic usage makes long-term treatment with these compounds undesirable (Rutgeerts *et al.*, 1995, 2005). A meta-analyses of 5-ASA in preventing post-operative recurrence demonstrated a mild effect (Akobeng *et al.*, 2016). The efficacy of azathioprine or 6-mercaptopurine has been inconsistent (Peyrin-Biroulet, Deltenre, *et al.*, 2009; Malireddy *et al.*, 2010). The use of biologic therapies, namely anti-TNF therapies, post-operatively appears promising (Singh *et al.*, 2015)s. The timing of initiation of biologic treatment seems to be critical. A Japanese study compared two groups of CD patients that were treated with infliximab following surgery (Suzuki and Yamada, 2012). The first group started infliximab at the time of post-surgical recurrence; the second group started infliximab immediately after surgery. Remission rates at 1 year were 57% in the group that waited for recurrence to initiate treatment and 92% in the

group that started therapy immediately post-operatively. This pattern persisted through 3 years of follow-up. These results suggest that the immediate post- operative period leaves the gut in a state that is more amenable to adjuvant therapy. However, due to the perceived risks associated with starting potent immunosuppressive therapies immediately following surgery, there is some reluctance to place patients on these medications soon after surgery. Many clinicians prefer to wait up to six months post-operatively before initiating treatment.

Other exposures, less well-studied than smoking and traditional medical therapies, offer alternative pathways to modulate the course of post-operative recurrence. These factors include dietary composition, the microbial milieu of the gut, and the consequent metabolic products of luminal bacteria. Diet, specifically enteral nutrition, has been shown to be important in preventing complications in CD patient's undergoing surgery (Y. Li *et al.*, 2015). In the post-operative period, supplemental enteral feeds while sleeping were associated with decreased recurrence at one year (Yamamoto *et al.*, 2007). A 2009 meta-analysis suggested that probiotics were an underwhelming therapy to decrease post-operative CD recurrence (Doherty *et al.*, 2009). Since then, a promising probiotic formulation, VSL#3, has demonstrated anti-inflammatory effects in the post-operative period (Fedorak *et al.*, 2015). In the randomized controlled trial assessing the efficacy of VSL#3 in the post-operative period 119 patients were randomized to probiotic or placebo, and although severe endoscopic recurrence rates did not differ significantly at 90 days or one year, those receiving VSL#3 had decreased mucosal inflammatory cytokine levels. Prebiotics have not been tested as a mono-therapy in post-operative CD recurrence, but

have been trialed, unsuccessfully, in combination with probiotics (when paired together they are dubbed a synbiotic) (Chermesh *et al.*, 2007).

An insight into the bacterial population associated with post-operative CD recurrence can be gained by examining three mouse studies. First, Rigby *et al.* demonstrated that IL10 -/- mice develop inflammation and fibrosis post-ICR, though a germ-free environment prevents the inflammatory response (Rigby *et al.*, 2009). Devine *et al.* followed this work by detailing the microbial changes in wild-type mice following ICR, and found that ICR reduced species diversity and richness, and saw the predominant bacterial phylum become Firmicutes, with a minor contribution from Proteobacteria (Devine *et al.*, 2013). Perry *et al.* attempted to modify the microbiota following ICR using fecal transplant (Perry *et al.*, 2015). This study noted an increased bacterial load in the small intestine of post-ICR mice. Fecal transplant was successful in reintroducing the phylum Bacteroidetes, but was unable to restore microbial diversity or improve inflammation.

Human studies examining the bacterial changes following ICR for CD reveal similar trends to the aforementioned mouse work. Increased bacterial growth is present following ICR (Neut Bulois, P., Desreumaux, P., Membre, J. M., Lederman, E., Gambiez, L., 2002). Bacterial community instability and decreased community robustness is seen in CD patients post-ICR who experience recurrence (Dey *et al.*, 2013; De Cruz, Kang, *et al.*, 2015; Mondot *et al.*, 2015). Specific bacterial populations have been associated with recurrence following ICR, but the nature of these studies does not enable the establishment of a cause-effect relationship.

1.5.3 Difficulties associated with the study of post-operative Crohn's disease

Although the potential benefits and importance of studying of post-operative CD recurrence has been recognized, the logistical challenges regarding study design has made research very challenging. First, in human populations, lengthy patient follow-up is needed to reach the relevant endpoints. Literature suggests that to ensure a 50% recurrence rate one must wait up to a year, and that number is derived from patients that are not prescribed any medical therapy post-operatively (Buisson *et al.*, 2012). Given the fact that a number of different drug classes have been shown to prolong remission in the setting of post-operative maintenance therapy, the timeframe to achieve recurrence might be even longer than anticipated. This problem is somewhat tempered by the fact that endoscopically, even patients with a prolonged clinical remission, likely still demonstrate an element of disease (Sorrentino and Paviotti, 2009).

Next, CD patients undergo surgery for a number of different reasons, ranging from perforation to fibrostenosis. These different presentations may very well be associated with a different microbiota and different immune profiles. This leads to the question of whether it is most appropriate to focus on a single disease phenotype, ignore disease phenotype altogether, or to assess whether the phenotypes differ via subgroup analysis once all subjects have been recruited. This final approach may lead to studies lacking adequate power after subject recruitment.

Third, following surgery, medical manipulation may obfuscate the natural course of CD recurrence. It is not ethical to withhold treatment, and due to the variety of medical therapies and treatment approaches, it is very difficult to standardize patient care.

Finally, the assumption that post-operative CD recurrence is similar to the initial development of CD is unproven. The inciting mechanism and corresponding microbiota may not bear any resemblance to one another. Nonetheless, post-operative CD recurrence justifies study, even if it may not be more broadly applicable to other CD patients.

1.6 Mouse models of Crohn's Disease

1.6.1 Medical mouse models of Crohn's Disease

The use of animals as a model for human health and disease stretches back to ancient Greece, where Aristotle studied the embryogenesis of chicks, through the Renaissance, which saw William Harvey detail the cardiovascular system of numerous animals, to today, where animal models are used for the study of innumerable diseases (Ericsson, Crim and Franklin, 2013). In this regard IBD and CD are no exception. Numerous models exist that target the pathophysiologic causes of the disease, addressing the mucosal barrier, expression of inflammatory mediators, microbial milieu, and the adaptive and innate immune systems. These models can be classified by the mechanism by which they induce colitis, and include chemically-induced models, transgenic models, and genetically-engineered models.

First, there are animal models used in CD research where the disease is induced chemically. This include the 2,4,6- Trinitrobenzene sulfonic acid (TNBS) model. This model represents both UC and CD, but is more representative of CD (Motavallian-Naeini *et al.*, 2012). Animals subjected to TNBS treatment develop a hypersensitivity reaction resulting in intestinal inflammation following administration of the molecule.

Indomethacin-induced enterocolitis is another model of chemically induced CD-like disease. Administration of indomethacin causes a inhibition of the synthesis of protective prostaglandins, which is exacerbated by luminal microbes (Jurjus, Khoury and Reimund, 2004).

Transgenic mice represent another class of animal models for CD. Two transgenic models include STAT4 and HLA-B27. STAT4 transgenic mice develop symptoms resembling CD, including colitis, diarrhea and weight loss (Simpson *et al.*, 1998). STAT4 is typically involved in the regulation of T-helper cells. HLA-B27 transgenic mice develop inflammation throughout the entire gastrointestinal tract mediated by mononuclear immune cells (Rath, Wilson and Sartor, 1999).

Gene-knockout mice are a popular choice for modeling CD. These include NOD2 knockout, IL-23 knockout, and IL10 knockouts, among others. NOD2 mutation has been noted to be associated with the development of CD, and murine mutant models possess immunologic deficits that result in CD-like disease (Goyal *et al.*, 2014). The IL-23 knockout develops mucosal inflammation specifically, through altered regulation of TH17-produced cytokines (Lees *et al.*, 2011; Goyal *et al.*, 2014). Finally, IL-10 knockout mice serve as the basis for many animal experiments in CD. This model was originally noted to develop spontaneous and chronic enterocolitis in 1993 (Kühn *et al.*, 1993). IL-10 is an anti-inflammatory cytokine that regulates the response to commensal bacterial and antigens (Unutmaz and Pulendran, 2009). The IL-10 knockout mouse is pertinent to the case of post-operative CD, as it has been shown to develop inflammation and fibrosis following ICR (Rigby *et al.*, 2009).

1.6.2 Surgical mouse models of Crohn's Disease

The first use of ICR in a murine model was performed to analyze short gut syndrome (Helmrath *et al.*, 1996, 1998). These studies laid the groundwork for the murine model of ICR, establishing the need for liquid diet in the peri-operative period, and the use of 9-0 monofilament suture material in creation of the anastomosis. This work opened the door to the development of an ICR mouse model for CD using the IL-10 knockout mouse. Following ICR, IL-10 knockout mice, but not wildtype mice, develop inflammation and fibrosis in the neo-terminal ileum (Rigby *et al.*, 2009). The development of post-ICR fibrosis in this model has been confirmed, along with systemic immune activation (Borowiec *et al.*, 2012). Subsequent work has attempted to delineate the bacterial changes following ICR. This work has revealed marked shifts, especially in Clostridial species, along with a loss of diversity and richness (Devine *et al.*, 2013). An attempt at modulating inflammation in this model modified the microbiota using fecal transplant post-ICR was successful in reducing ileitis, but worsened colitis (Perry *et al.*, 2015). Further exploration of this model will be facilitated by a recently published standardized operative procedure (Perry *et al.*, 2014).

1.7 Aims and hypothesis

The goal of this project was to identify genetic, immunologic, and microbial factors that are associated with the post-operative recurrence of CD, with the hypothesis that certain patient and environmental factors lead to post-operative disease recurrence in the CD patient.

Table 1.1: Comparison of IBD subtypes, CD and ulcerative colitis

	CD	UC
COMMON SYMPTOMS	Diarrhea, fever, abdominal pain, fatigue, weight loss, loss of appetite	Bloody diarrhea, fever, abdominal pain, fatigue, weight loss, loss of appetite
DISTRIBUTION	Patchy disease anywhere in the gastrointestinal tract	Continuous inflammation starting from the rectum and extending proximally. Confined to the colon
PERIANAL DISEASE	Common	Rare
DEPTH OF INFLAMMATION	Full-thickness, transmural	Mucosal
GRANULMOAS	Commonly found on biopsy	Rare
FISTULIZATION	Common	Never
STENOSIS	Common	Rare
SMOKING	Smoking increases risk	Smoking decreases risk

Adapted from the CCFC Impact Report

Table 1.2: Endoscopic recurrence scoring system

RUTGEERTS SCORE	
0	No lesions
1	< 5 aphthous lesions
2	>5 aphthous lesions with normal mucosa between the lesions, or skip areas of larger lesions or lesions confined to the ilecolonic anastomosis
3	Diffuse aphthous ileitis with diffusely inflamed mucosa
4	Diffuse inflammation with already large ulcers and/or narrowing

From Rutgeerts *et al.* (1990)

Chapter 2: Endospore forming bacteria are associated with maintenance of surgically-induced remission in Crohn's disease

Abstract

Background and Aim: Crohn's disease (CD) patients who undergo ileocolonic resection (ICR) typically have disease recurrence at the anastomosis, which has been linked with a gut dysbiosis. The aims of this study were to define the mucosa-associated microbiota at the time of ICR and to determine if microbial community structure at the time of surgery was predictive of future disease relapse.

Methods: Ileal biopsies were obtained at surgery and after 6 months from CD subjects undergoing ICR (n=45). Composition and function of mucosal-associated microbiota was assessed by 16S rRNA sequencing and PICRUSt analysis. Endoscopic recurrence was assessed using the Rutgeerts score. Linear discriminate analysis effect size (LEFse) was used to evaluate differential bacterial abundance and potential biologic significance.

Results: At 6 months, 30 subjects remained in remission while 15 had recurrent disease. At the time of surgery, LEFse analysis of mucosal biopsies showed that the Clostridiales order predicted maintenance of remission whereas Enterobacteriales predicted disease recurrence ($p<0.05$). An increase in the endospore-forming Lachnospiraceae from surgery to 6 months post-ICR was associated with remission ($p=0.01$). At the time of surgery, a ratio of 3:1 between anaerobic endospore-forming bacterial families to families capable of aerobic respiration within the Firmicutes phylum was shown to be predictive of maintenance of remission in an adjusted analysis (OR-9.2 95% CI 1.8-47.7 $p<0.01$).

Conclusions: Gut recolonization following ICR is facilitated by microbes, which are capable of either aerobic respiration or endospore formation. The relative proportions of these species at the time of surgery is predictive of subsequent microbial community restoration and disease recurrence.

Introduction

Crohn's disease (CD) involves transmural inflammation of the alimentary tract (Abraham, Cho and Abraham, Clara, Cho, 2009). Nearly 50% of patients require an intestinal resection within 10 years of diagnosis, typically for intestinal stricturing, enteric fistula, abscess formation, or inflammation that failed to respond to medical treatment (Frolkis *et al.*, 2013; Schlussel, Steele and Alavi, 2016). The most common type of resection for CD patients is an ileocolic resection (ICR) with anastomosis, which involves removal of diseased areas involving the last part of the small intestine and a portion of the right colon with ileocolonic anastomosis between healthy bowel loops (Fowler *et al.*, 2014). Unfortunately, disease recurs in up to 85% of patients within one year of their resection (Connelly and Messaris, 2014) and 25% of patients require a second bowel operation within 5 years of the first surgery (Frolkis *et al.*, 2014). The neo-terminal ileum, or upstream portion of anastomosis, is the most common location in which the disease recurs (Rutgeerts *et al.*, 1984). In 1991, Rutgeerts et al. reported that proximal fecal diversion prevented post-operative recurrence of CD (Rutgeerts *et al.*, 1991). The relationship with the gut microbiota was reinforced in a murine model of post-surgical bowel inflammation, where a germ-free environment prevented the inflammatory response (Rigby *et al.*, 2009). Taken together, it is clear that the fecal stream, and its associated contents, plays a central role in disease recurrence; however, the microbial constituents leading to postoperative recurrence remain elusive.

A number of studies have evaluated the microbiota at the time of surgery in attempts to identify bacterial signatures that predict remission or relapse (Neut Bulois, P., Desreumaux, P., Membre, J. M., Lederman, E., Gambiez, L., 2002; Darfeuille-Michaud

et al., 2004; Sokol *et al.*, 2008; Dey *et al.*, 2013; De Cruz, Kang, *et al.*, 2015; Mondot *et al.*, 2015; Wright *et al.*, 2016). In these studies, members of the Firmicutes and Proteobacteria phyla have been the main taxa identified in prediction of remission and recurrence. However, the influence of intestinal microbiota on post-operative recurrence is still incompletely understood. Intestinal resection constitutes a large insult to the resident microbiota and re-colonization depends upon which commensal organisms are able to survive and reproduce following surgery. Survival of organisms under such stress would require either the ability for aerobic respiration or alternatively the ability to sporulate during the insult. In murine models of ICR, Firmicutes dominate post-operatively (Devine *et al.*, 2013; Perry *et al.*, 2015). This is not surprising given the robust phenotypic diversity of this phylum that comprises both aerobes, facultative and strict anaerobes, and species capable of forming endospores. Therefore, the pre-operative community composition within the Firmicutes phylum may determine how recolonization proceeds following surgery and thereby modulate the overall inflammatory environment in the post-operative gut. This study involved a prospectively collected cohort of post-ICR CD subjects. The aims were to define the mucosa-associated microbiota at the time of ICR and at 6 months following ICR, and to determine if the bacterial community structure at the time of surgery was predictive of future disease relapse. Herein, we show that the balance at the time of surgery between groups of bacteria capable of either aerobic respiration or endospore formation was predictive of future disease relapse.

Methods

Patient Cohort

The study was approved by the Health Research ethics board at the University of Alberta (Pro00028147) and was carried out between June 2012 and June 2015. Subjects (n=45) were recruited and consented by ML, TP or LD at the University Hospital, Royal Alexandra Hospital, Grey Nuns Hospital, or Misericordia Hospital prior to their operation. Exclusion criteria included antibiotics within one month of surgery, creation of a diversion ostomy, age less than 18 years old, inability to consent, and women who were pregnant or wishing to become pregnant. 6 months post-operatively endoscopic remission was assessed prospectively by the treating gastroenterologist using the validated Rutgeerts score with recurrence being defined as ≥ 2 (Rutgeerts *et al.*, 1984). All subjects received intravenous antibiotics immediately pre-operatively as defined by our institutions protocol (cefazolin and metronidazole or clindamycin and gentamycin in cases of β-lactam allergy). On the day of surgery and at post-surgical ileocolonoscopy, the subject's medical record was reviewed by research coordinators. Subjects were phenotyped based on the Montreal Classification as per the following criteria: age at diagnosis; disease location; and disease behavior(Silverberg *et al.*, 2005) . Post-operative therapy was determined by the subject's clinical gastroenterologist.

Sample collection

Mucosal biopsies were obtained at the time of resection from macroscopically normal tissue in the ileum. At 6 months post-op, biopsies from non-ulcerated tissue in the

neo-terminal ileum were obtained from within 5 cm of the anastomosis. All samples were snap frozen in liquid nitrogen and stored at -80ºC.

Assessment of tissue inflammation

Snap frozen biopsies were homogenized in PBS containing 0.05% Tween 20. Homogenates were centrifuged at 10 000 rpm for 10 minutes and the supernatant was retained to measure tissue Interleukin(IL)-2, IL-6, IL-8, and TNFα using a Meso Scale discovery system (Meso Scale Diagnostics, Gaithersburg, MD, USA) as per the manufacturer's protocol.

Microbial Composition

The FastDNA Spin Kit (MP Biomedicals, OH USA) was used to extract total genomic DNA as per the manufacturer's protocol. DNA was quantified using PICO green assay. 10ng was used for sequencing of the 16s ribosomal RNA gene using 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 355R (5′-GCT GCC TCC CGT AGG AGT-3′) universal primers targeting the V1-V2 region (Lane *et al.*, 1985). A PGM Ion Torrent sequencer (Thermo Fisher) was used to sequence the samples. Amplicons were purified using Agilent magnetic beads. Sequencing reads greater than 250 basepairs were selected to obtain high quality reads. After trimming for quality, a total of 1 320 578 reads were obtained from 70 ileal samples (n= 38 pre-operative samples and n= 32 post-operative samples). The number of sequence reads ranged from 617 to 56 192 with a median value of 18 772. Sequences were subsequently analyzed using RDP11 and QIIME pipelines using UCLUST to assign sequences, ChimeraSlayer was used for chimera detection and removal. Alpha diversity was analyzed on the OTU table rarefied

to 617 reads using the Shannon diversity index. Prediction of metagenome functional content was developed using PICRUSt software and PICRUSt predictions were categorized as level 1 to 3 into KEGG pathways (Langille *et al.*, 2013).

Data analysis

Statistical analysis was performed using STATA, Galaxy, and QIIME software. Demographic and cytokine data was assessed using Student's t-test for continuous variables after ensuring normality assumptions were met using the Shapiro-Wilk test, and Fisher's exact test for categorical variables. Adjustment was performed using a multivariable logistic regression including factors that achieved a p-value <0.1 on univariate analysis. Microbial comparisons were assessed using Kruskal-Wallis one-way test of variance on taxa present in greater than 75% of all samples. Testing of this kind incurs a substantial multiple comparison burden which we have corrected for false discovery rate (FDR) using the Benjamini-Hochberg procedure. Results where $p \leq 0.05$ following FDR-adjustment will be referred to as "significant following FDR-adjustment", whereas those results with a p-value ≤ 0.05 and an FDR-adjusted p-value > 0.05 will be referred to as "significant." To identify factors with differentiating abundance in the different groups, the LDA (Linear Discriminant Analysis) Effect Size (LEfSe) algorithm was used with the online interface Galaxy (http://huttenhower.sph.harvard.edu/galaxy/root). The algorithm is designed to evaluate differential abundance along with potential biologic significance and involves the non-parametric factorial Kruskal-Wallis (KW) sum-rank test to identify populations with significant differential abundance between groups, an (unpaired) Wilcoxon rank-sum test to assess a biological significance and a Linear Discriminant Analysis the calculates the

effect size of the differentially abundant feature. P values were set at 0.05 without FDR-adjustment and a linear discriminant analysis cut-off score of 3.0 (Segata *et al.*, 2011). Partial least square analysis discriminant analysis (PLS-DA) was applied to cluster observations with similar microbial profiles and to identify taxa that led to a discrimination between remission and recurrence. Data is presented as mean value ± the standard deviation (SD).

Results

Subject demographics were similar in those who remained in remission and in those who recurred

Subject demographics are detailed in Table 2.1. Of 45 subjects, 30 remained in endoscopic remission at 6 months while 15 had recurrent disease. Clinical factors that have previously been associated with post-operative disease activity did not significantly differ between subjects who recurred and those who remained in remission including disease location, smoking status, and the presence of peri-anal disease. The proportions of remission and relapsing subjects using each class of CD medications, including antibiotic usage in the immediate post-operative period, was not significantly different at the time of surgery or at the time of follow-up ileo-colonoscopy.

The mucosal-associated microbial profile and its association with disease recurrence

Subjects that subsequently developed postoperative recurrence had a significantly elevated level of Enterobacteriaceae (Remission: 16.9 (5.6%) Recurrence: 44.3 (10.4%) p=0.03, FDR-adjusted p=0.11), and a significant reduction in Clostridiales (Remission: 19.5 ± 3.6% Recurrence: 8.8 ± 2.1%, p=0.02, FDR-adjusted p=0.10) at the time of surgery when compared to subjects that remained in remission (Table 2.2). LEfSe algorithm revealed two candidate orders (Clostridiales, Burkholderiales) that potentially predicted remission versus one candidate order, Enterobacteriale, associated with recurrence (Figure 2.1).

When analyzing changes that occurred within subjects between the time of surgery and six months post-ICR, differences were observed in those subjects who remained in

remission and those which relapsed (Table 2.2). Recurrent subjects experienced a significant elevation in the proportion of reads associated with the Bacteroidaceae family (Recurrent subjects at surgery: 29.5% (26.9%), Recurrent subjects at follow-up: 56.7% (31.0%) p=0.05, FDR-adjusted p=0.16). In contrast, subjects who remained in remission demonstrated a significant after FDR-adjustment increase in the family Lachnospiraceae, which was not equaled in the recurrent group (Remission subjects at surgery: 8.5% (6.5%), Remission subjects at follow-up: 21.1% (18.7%) p=0.01, FDR-adjusted p=0.04).

At the time of surgery α-diversity was similar in subjects that remained in endoscopic remission as compared to those with disease recurrence at 6 months (Shannon index: Remission: 4.10 (0.87) Recurrence: 3.99 (0.83)). α-diversity within an individual was not significantly affected by surgery (Shannon index: Surgery: 4.17 (0.86), Month 6: 3.88 (0.86)).

Aerobic respiration, sporulation and germination as predictors of disease recurrence

PICRUSt was used to infer functionally important metabolic functions in mucosal-associated microbiota (Figure 2.2). When analyzing microbial function at the time of surgery, it was noted that the mucosal-associated microbiota in subjects which remained in remission possessed a higher proportion of reads associated with sporulation and germination (Figure 2.3). In contrast, samples from subjects experiencing recurrence collected at endoscopy contained a greater proportion of reads associated with the citrate

cycle (i.e. aerobic respiration) while those samples collected from subjects in remission contained a higher proportion of reads associated with the germination of spores.

Anaerobic spore-forming bacteria are associated with a maintenance of remission following ICR when compared to the relative proportion of aerobic bacteria

Based upon our findings showing germination of spores to be associated with remission while aerobic respiration was associated with disease recurrence, we examined the relationship within the Firmicutes phyla between families capable of aerobic respiration and anaerobic endospore-forming bacterial families. We defined each family on the basis of any contained species and these characteristics (Table 2.4). Subsequent analysis revealed a relationship between recurrence and the ratio of anaerobic endospore-forming bacterial families to families capable of aerobic respiration present at the time of surgery. Overall, subjects that remained in remission had an increased ratio of anaerobic spore-forming bacteria to bacteria capable of aerobic respiration compared with subjects that had disease recurrence (Figure 2.3A). A ratio greater than 3:1 between these two groups of bacteria was identified in 26 subjects, 21 of which remained in endoscopic remission. In the 12 subjects where the ratio was less than 3:1, 8 experienced recurrence. This represents an unadjusted odds ratio of 8.4 (95% CI 1.8-39.4 $p<0.01$). After multivariable adjustment the odds ratio maintained significance (OR-9.2 95% CI 1.8-47.7 $p<0.01$). In addition, a ratio of >3:1 at the time of surgery was associated with increased α-diversity at follow-up endoscopy (High ratio: 4.10 (0.79), Low ratio: 3.36 (0.69) $p=0.04$).

Recurrence and endospore content are independent of mucosal inflammation

To determine if differences in mucosal microbial composition and function were related to the degree of inflammation, levels of IL-2, IL-6, IL-8, and TNFα were measured in biopsies taken at the time of surgery. None of these cytokines were associated with disease remission at six months (Figure 2.4). In addition, levels of each cytokine showed no correlation with the different ratios of anaerobic spore-forming bacteria compared to bacteria capable of aerobic respiration, suggesting that this microbial signature was inflammation independent. As a validation measure, we assessed the level of TNFα in those receiving pre-operative anti-TNF therapy, compared to those not on anti-TNF therapy, and there was a trend toward reduced mucosal levels of TNFα at the time of surgery in those on the biologic therapy (Figure 2.4).

Discussion

In this study, we report an association between mucosal-associated microbiota at the time of surgery and post-operative recurrence of CD in a cohort of post-operative CD patients. We also identify, for the first time, the potential impact of endospore-forming bacteria in the process of gut recolonization and post-operative CD recurrence. Finally, we propose a novel niche driven approach to the assessment of the microbiota at the time of intestinal resection.

The majority of colonic microbiota are made up of obligate anaerobic bacteria. Major physiologic stressors, such as intestinal surgery, can induce massive microbial ecological shifts (Guyton and Alverdy, 2017) and survival of local species depends on their ability to cope with these stressors. A number of factors are likely to influence bacterial populations following ICR including increased oxygen stress at the time of surgery, retrograde flow of colonic contents following removal of the ileocecal valve, ongoing inflammatory changes involved in intestinal wound healing, and altered local and systemic immune responses following surgery (Alazawi, Pirmadjid and Lahiri, 2016; Guyton and Alverdy, 2017). Bacterial survival mechanisms include the genetic ability to deal with oxygen in the form of aerobic respiration as well as the ability to produce endospores; indeed over 50% of gut microbes may be capable of producing endospores (Browne *et al.*, 2016). Endospores allow bacteria to persist in hostile environments, including those with high atmospheric oxygen (Browne *et al.*, 2016). Here we demonstrate that endospore formation may be a viable strategy for survival in the post-operative gut following intestinal resection. The increased relative fitness of endospores post-operatively may partially explain the predilection for *C. difficile* infection following bowel

resection compared to other intra-abdominal surgeries (Zerey *et al.*, 2007). However, not all spore-forming bacteria carry the pathologic implications of C. difficile, and recent evidence suggests that there may be a beneficial transfer of spore-forming bacteria between individuals living in close contact (Schloss *et al.*, 2014). Administration of endospores has also been shown to recruit T-regulatory cell populations (Atarashi *et al.*, 2011). Importantly, many anaerobic bacteria capable of forming spores (e.g *Clostridiaceae*, *Lachnospiraceae*) also produce short-chain fatty acids, which have been shown to be beneficial to intestinal health (Tan *et al.*, 2014). Interestingly, a lack of *Faecalibacterium prausnitzii* at the time of surgery has been identified to be associated with disease recurrence (Sokol *et al.*, 2008; Wright *et al.*, 2016). F. prausnitzii are important butyrate-producing bacteria but are highly oxygen sensitive (Duncan *et al.*, 2002) explaining their reduction in patients with active inflammation. A recent study has identified the presence of more than 30 genes related to endospore formation in the genome of *F. prausnitzii* (Caspi *et al.*, 2016), suggesting that it has the ability to withstand severe insults, such as intestinal surgery, through the formation of endospores.

It has been challenging to define specific gut microbial populations that may predict health or disease in an individual (Mariat *et al.*, 2009; Sha *et al.*, 2013). Surgery induces a large insult and we propose that two populations within the Firmicutes phylum- first, those capable of aerobic respiration, and second, those capable of endospore-formation- possess the capability to endure the operative insult and act as keystone species in the re-colonization of the gut. In agreement with previous studies (Neut Bulois, P., Desreumaux, P., Membre, J. M., Lederman, E., Gambiez, L., 2002; Darfeuille-Michaud *et al.*, 2004; Sokol *et al.*, 2008; Dey *et al.*, 2013; De Cruz, Kang, *et al.*, 2015; Mondot *et*

al., 2015; Wright *et al.*, 2016), we show that at 6 months following surgery the microbial composition differs between patients in remission and those who have disease recurrence. This is expected as the presence of inflammation alters gut microbial composition (Winter *et al.*, 2013; Winter, Lopez and Bäumler, 2013) and thus a causative relationship cannot be determined. However, we speculate that the relative amounts and presence of specific species at the time of surgery may determine how subsequent colonization occurs. In agreement with previous studies, we found that the gut microbiota at the time of surgery differed between patients who remained in remission versus those which suffered early disease relapse. In a small cohort, De Cruz (De Cruz, Kang, *et al.*, 2015) identified patients who relapsed to have a greater relative amount of Streptococcaceae and Enterococcacea and a relative lack of Clostridiales and Bacteroidales orders at the time of surgery. Further, at the time of surgery, patients who remained in remission at 6 months had higher amounts of Faecalibacterium and Ruminococcus, both putative spore-formers. In a similar study, Wright et al (Wright *et al.*, 2016) also identified a reduced amount of F. prausnitzii and increased levels of Proteus, a member of Enterobacteriaceae to be increased. In that Streptococcaceae and Enterococcacea are capable of aerobic respiration and are non-spore formers, overall these findings are in agreement with our hypothesis that relative ratios of these functional groups may be the determining factor in gut recolonization and subsequent disease recurrence. Interestingly, recolonization with encapsulated endospores from selected Firmicutes species was recently demonstrated to be effective in preventing recurrence of *C. difficile* infection (Khanna *et al.*, 2016).

There are a number of limitations to be acknowledged in this work. First our cohort did not receive standardized post-operative care. This led to heterogeneity in terms of anti-inflammatory and antimicrobial regimens, which were underpowered for sub-group analysis. Also, no subjects suffered from a severe recurrence (Rutgeerts Grade 4).

In conclusion, we demonstrate, for the first time, that a dominance of endospore-forming anaerobic bacteria in the ileal mucosa at the time of surgical resection is associated with maintenance of disease remission, whereas a dominance of aerobic bacteria is associated with disease recurrence. These findings suggest that a possible strategy to prevent post-operative recurrence of CD is to promote recolonization with encapsulated endospores from selected Firmicutes species. Overall, these findings highlight the importance of overall microbial ecosystem structure in gut health. Gaining an understanding of how microbial communities re-assemble following gut insults has the potential to aid in the development of specific microflora-altering therapy aimed at modulating this process and has profound clinical implications.

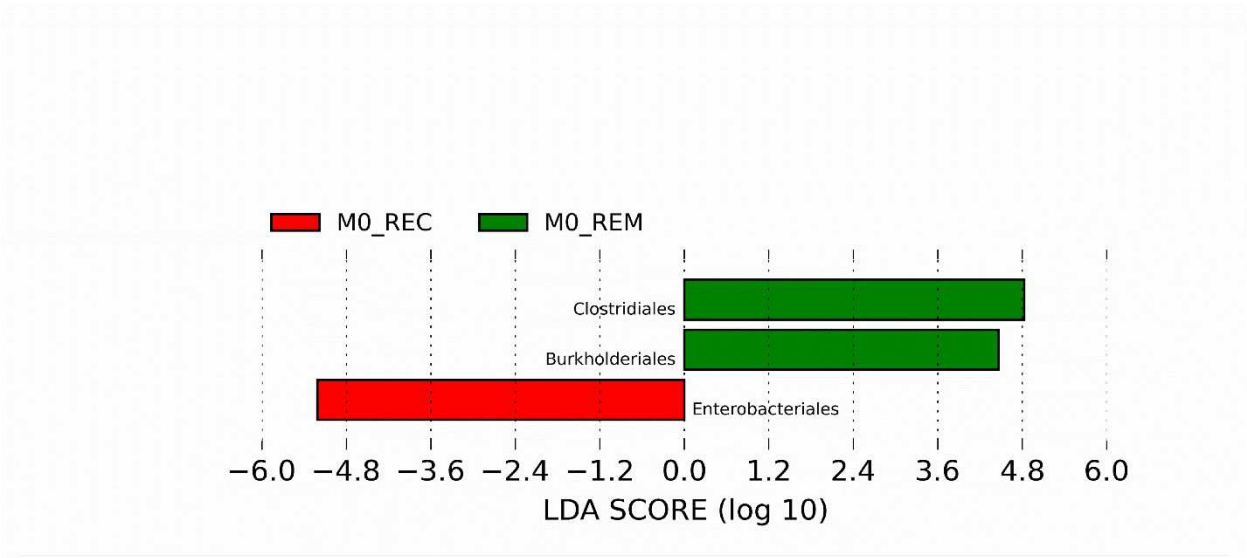


Figure 2.1. Linear Discriminant Analysis Effect Size (LEfSe) analysis of taxonomic data at baseline. LEfSe analysis showed that groups of microbes differed at the time of surgery. Driving the separation were Enterobacteriales in the recurrence cohort and Clostridiales and Burkholderiales in the remission cohort. The relative abundance of OTUs at the time of surgery associated with bacterial families were evaluated using a LEfSe algorithm in regard to predicting recurrence.

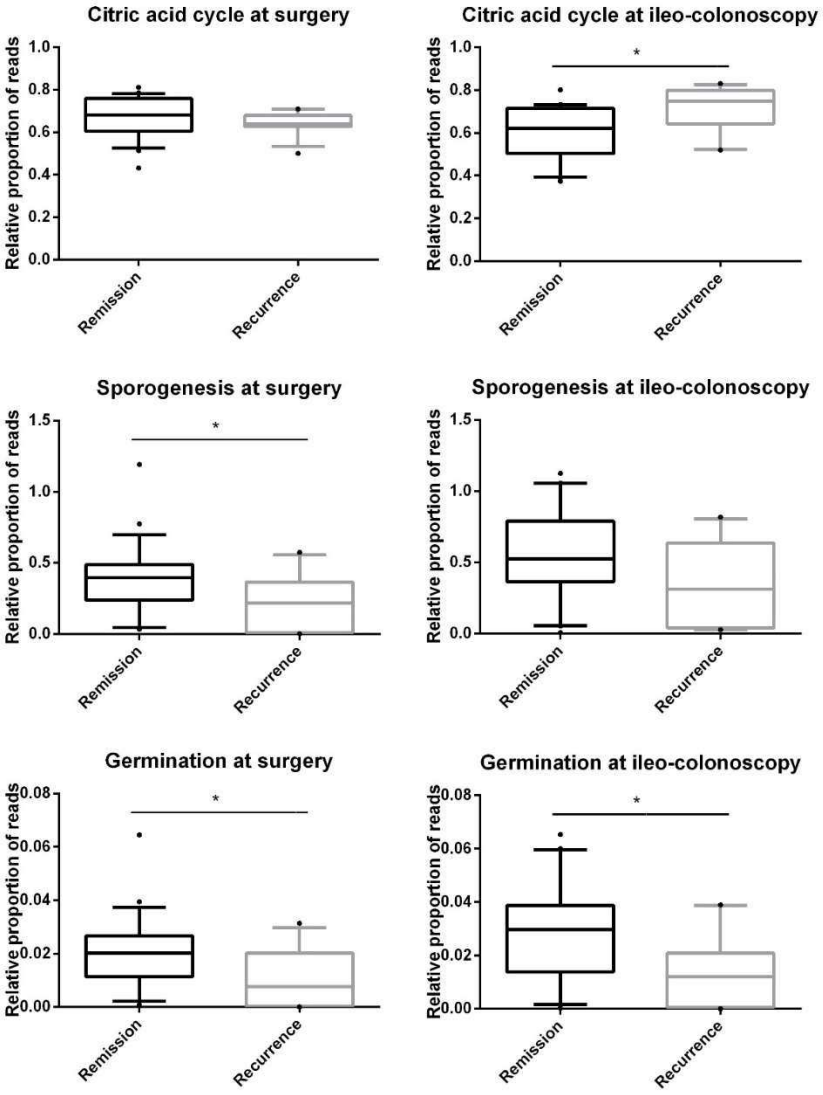


Figure 2.2. PICRUSt analysis of mucosal-associated microbiota showing relative abundance of specific functional pathways associated with bacterial sporogensis, germination, and aerobic metabolism. At the time of surgery, samples taken from subjects who remained in remission had higher levels of sporogenesis (b) and germination (c) compared with samples taken from subjects who had disease recurrence. This remained higher at the time of ileo-colonoscopy. In contrast, at endoscopy, those subjects which had disease recurrence had increased proportion of genes related to aerobic respiration (citric acid cycle) (a). * $p<0.05$

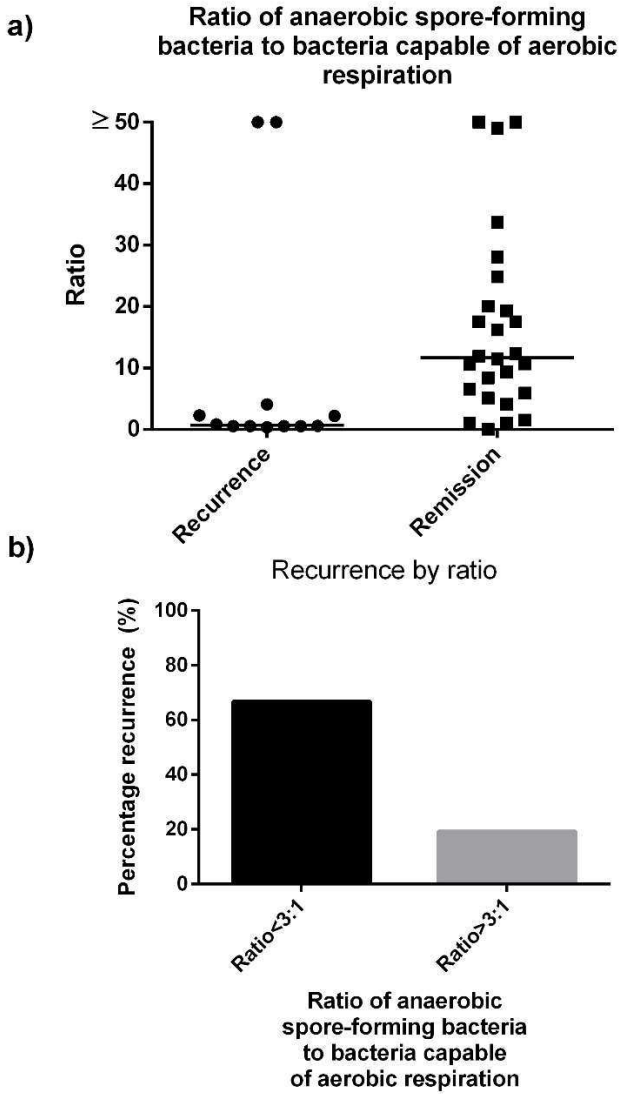


Figure 2.3. Ratio of obligatory anaerobic spore-forming bacteria to those capable of aerobic respiration within the Firmicutes phylum at the time of ICR. (a) Median ratio within the MAM of specimens collected at the time of ICR of obligatory anaerobic spore-forming families to families capable of aerobic respiration within the Firmicutes phylum (b) Rate of recurrence (%) in those with a ratio of >3:1 and <3:1 in terms of anaerobic endospore-forming bacteria and those bacteria capable of aerobic respiration within the Firmicutes phylum. The rate of recurrence in those with a ratio of >3:1 was 19% and was 67% in those with a ratio <3:1.

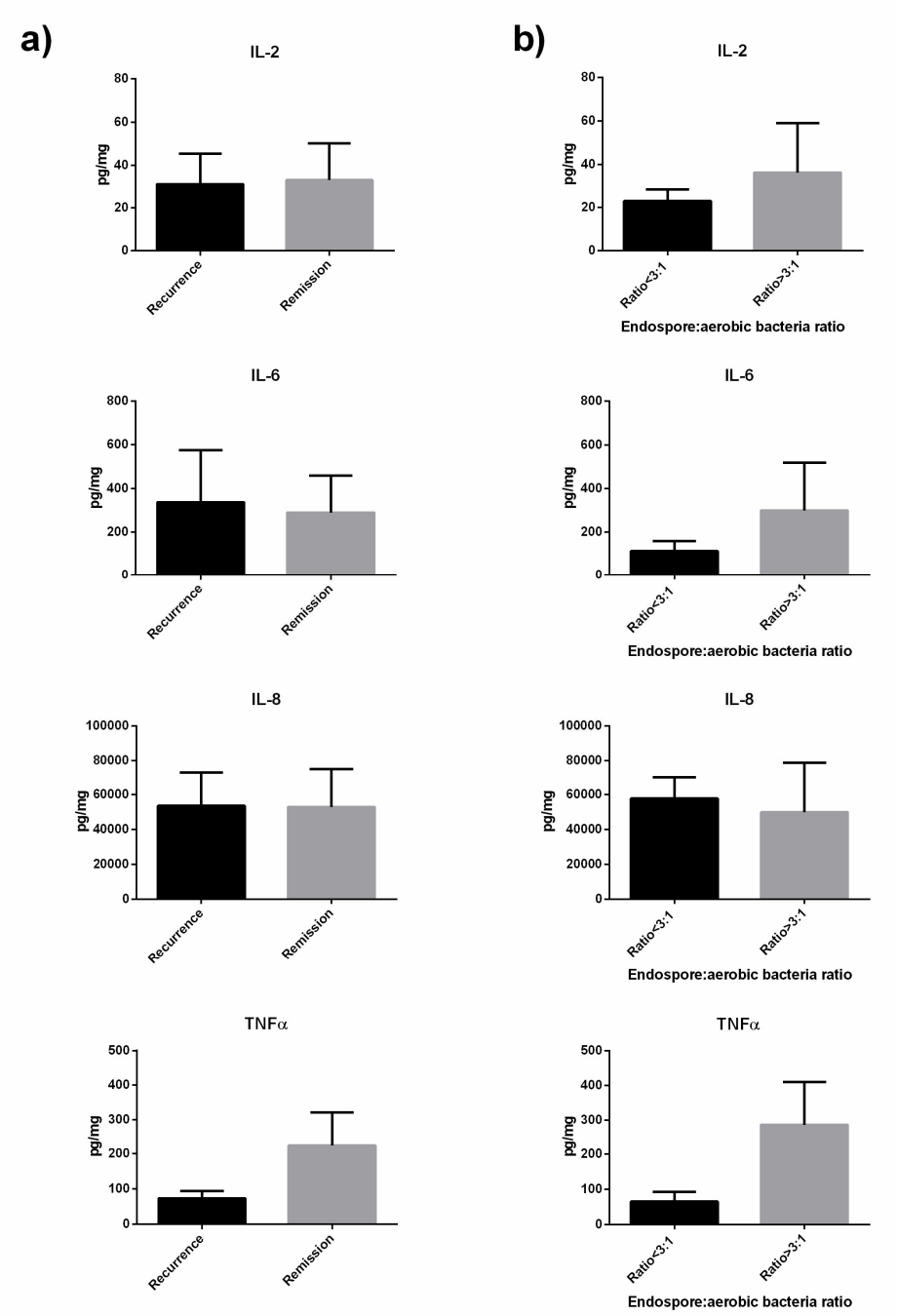


Figure 2.4: Mucosal inflammatory cytokines at the time of surgery. None of the cytokines that were measured from the mucosal ileal specimens collected at the time of surgery were associated with cytokines were associated with a) recurrence status or b) endospore content.

Table 2.1: Clinical and demographic characteristics of subjects by recurrence status

	Recurrence (n=15)	**Remission** (n=30)	*p*
AGE (YEARS)	48.4	40.6	0.09
MALE (%)	20%	47%	0.11
PREVIOUS ICR (%)	60%	37%	0.21
AVERAGE TIME TO ENDOSCOPY (DAYS)	203	222	0.50
CURRENT SMOKER (%)	13%	10%	1.00
PERI-ANAL DISEASE (%)	13%	17%	1.00
DISEASE LOCATION			
ILEAL (%)	53%	73%	
COLONIC (%)	0	0	0.20
ILEOCOLONIC (%)	47%	27%	
DISEASE BEHAVIOUR			
INFLAMMATORY (%)	0%	7%	
STRICTURING (%)	60%	60%	0.88
PENETRATING (%)	40%	33%	
OPERATIVE INDICATIONS			
OBSTRUCTION (%)	93%	73%	
ENTERO-ENTERIC FISTULA (%)	7%	17%	0.36
FAILURE OF MEDICAL THERAPRY (%)	0%	10%	
AGE AT CD DIAGNOSIS			
LESS THAN 16 YEARS	7%	27%	
BETWEEN 16 AND 40 YEARS	80%	60%	0.35
GREATER THAN 40 YEARS	13%	13%	
PRE-OPERATIVE CD MEDICATIONS			
STEROID (%)	47%	27%	0.17
BIOLOGIC THERAPY (%)	53%	47%	0.76
5-ASA DRUG (%)	0%	20%	0.16
AZATHIOPRINE (%)	20%	47%	0.11
METHOTREXATE (%)	20%	7%	0.32
POST-OPERATIVE CD MEDICATIONS			
STEROID (%)	0%	7%	0.55
BIOLOGIC THERAPY (%)	47%	60%	0.55
5-ASA DRUG (%)	0%	17%	0.15
AZATHIOPRINE (%)	27%	40%	0.51
METHOTREXATE (%)	13%	3%	0.25
ANTIBIOTICS (%)	27%	47%	0.33

*p-value were calculated using Student's t-test for continuous variables. Categorical variables were assessed using the Fisher's exact test.

Table 2.2- Changes in bacterial populations from the time of surgery to follow up endoscopy in subjects with recurrence and remission.

Population	Surgical specimen in recurrent subjects (SD) n=12	6-month post-ICR specimen in recurrent subjects (SD) n=12	p-value	FDR-adjusted p-value	Surgical specimen in remission subjects (SD) n=26	6-month post-ICR specimen in remission subjects (SD) n=20	p-value	FDR-adjusted p-value
Phylum								
Proteobacteria	48.8% (35.0%)	19.1% (18.5%)	0.09	0.27	24.9% (25.4%)	24.9% (21.7%)	0.65	0.65
Bacteroidetes	32.3% (28.3%)	60.0% (31.3%)	**0.04**	0.16	46.6% (28.8%)	37.3% (32.1%)	0.28	0.65
Firmicutes	16.9% (17.6%)	20.1% (24.5%)	0.90	0.90	24.8% (21.5%)	35.9% (27.7%)	0.11	0.42
Actinobacteria	1.1% (0.9%)	0.6 (1.0%)	0.15	0.30	1.2% (1.7%)	0.9 (1.5%)	0.53	0.65
Class								
Bacteroidia	31.3% (29.1%)	59.2% (31.8%)	**0.05**	0.24	45.2% (32.4-58.1%)	36.1% (23.7-48.6%)	0.30	0.72
Clostridia	8.8% (7.4%)	18.8% (24.1%)	0.48	0.52	19.5% (18.2%)	32.2% (26.8%)	0.07	0.36
Gammaproteobacteria	45.6% (34.7%)	16.5% (18.2%)	0.09	0.27	19.8% (25.3%)	19.7% (18.3%)	0.36	0.72
Bacilli	5.9% (12.1%)	0.9% (1.4%)	0.06	0.24	4.8% (11.6%)	2.3% (4.1%)	0.97	0.97
Alphaproteobacteria	2.4% (2.0%)	1.9% (1.8%)	0.52	0.52	2.4% (5.0%)	3.5% (5.2%)	0.12	0.48
Order								
Bacteroidales	31.3% (29.1%)	59.2% (31.8%)	**0.05**	0.27	45.2% (29.3%)	36.1% (32.4%)	0.30	0.60
Clostridiales	8.8% (7.4%)	18.8% (24.1%)	0.48	1	19.5% (18.2%)	32.2% (26.8%)	0.07	0.33
Enterobacteriales	44.3% (35.9%)	16.0% (18.5%)	0.16	0.63	16.9% (25.0%)	16.8% (14.7%)	0.14	0.41
Rhizobiales	2.1% (2.0%)	1.9% (1.8%)	0.95	1	2.0% (3.8%)	3.4% (5.0%)	0.08	0.33
Lactobacillales	5.8% (12.0%)	0.9% (1.4%)	0.07	0.34	4.2% (10.3%)	2.0% (3.7%)	0.98	0.98
Burkholderiales	0.9% (1.4%)	0.5% (0.6%)	1.00	1	2.6% (3.1%)	0.6% (1.6%)	0.06	0.33
Family								
Bacteroidaceae	29.5% (26.9%)	56.7 (31.0%)	**0.05**	0.16	38.6% (27.1%)	34.5% (32.3%)	0.57	0.57
Enterobacteriaceae	44.3% (35.9%)	16.0% (18.5%)	0.16	0.31	16.9% (25.0%)	16.8% (14.7%)	0.14	0.41
Lachnospiraceae	4.6% (4.6%)	9.5% (13.1%)	0.46	0.46	8.5% (6.5%)	21.1% (18.7%)	**0.01**	**0.04**
Streptococcaceae	5.4% (11.4%)	0.4% (0.6%)	**0.05**	0.16	3.6% (9.4%)	1.5% (3.3%)	0.39	0.57

All values calculated using Kruskal-Wallis H-test. FDR- corrected q-values derived using Benjamini-Hochberg procedure.

Table 2.3: Bacterial populations following surgery.

Population	Surgery (SD) n=38	Month 6 (SD) n=32	p	FDR-corrected q-value
Phylum				
Proteobacteria	32.5% (30.8%)	22.8% (20.8%)	0.48	0.64
Bacteroidetes	42.1% (29.4%)	45.4% (33.6%)	0.64	0.64
Firmicutes	22.3% (20.7%)	30.3% (27.7%)	0.33	0.64
Actinobacteria	1.2% (1.5%)	0.8% (1.3%)	0.21	0.64
Class				
Bacteroidia	40.8% (30.0%)	44.3% (34.0%)	0.65	0.89
Clostridia	16.1% (16.4%)	27.4% (26.6%)	0.10	0.50
Gammaproteobacteria	28.0% (31.0%)	18.6% (18.3%)	0.89	0.89
Bacilli	5.1% (11.8%)	1.8% (3.5%)	0.29	0.87
Alphaproteobacteria	2.4% (4.3%)	3.0% (4.4%)	0.24	0.87
Order				
Bacteroidales	40.8% (30.0%)	44.3% (34.0%)	0.65	0.69
Clostridiales	16.1% (16.4%)	27.4% (26.6%)	0.10	0.44
Enterobacteriales	25.6% (31.6%)	16.5% (16.2%)	0.69	0.69
Rhizobiales	2.0% (3.3%)	2.9% (4.2%)	0.11	0.44
Lactobacillales	4.7% (10.9%)	1.6% (3.1%)	0.28	0.69
Burkholderiales	2.0% (2.8%)	1.2% (2.2%)	**0.05**	0.30
Family				
Bacteroidaceae	35.7% (27.4%)	42.4% (33.6%)	0.46	0.69
Enterobacteriaceae	25.6% (31.6%)	16.5% (16.2%)	0.69	0.69
Lachnospiraceae	7.2% (6.2%)	17.0 (17.8%)	**0.03**	0.12
Streptococcaceae	4.2% (10.1%)	1.1% (2.7%)	0.07	0.21

*All values calculated using Kruskal-Wallis H-test. Grey shading indicates a higher proportion in M6 specimens, no shading indicates higher in M0 specimens

Table 2.4: Classification of families within the Firmicutes phylum in terms of their member species ability to perform aerobic respiration or undergo sporogenesis

Class	Order	Family	Species capable of aerobic respiration	Spore-forming member species
Bacilli	Bacillales	Bacillales	Yes	Yes
		Listeriaceae	Yes	No
		Paenibacillaceae	Yes	Yes
		Staphylococcaceae	Yes	No
		Thermoactinomycetaceae	Yes	Yes
	Lactobacillales	Aerococcaceae	Yes	No
		Carnobacteriaceae	Yes	No
		Enterococcaceae	Yes	No
		Lactobacillaceae	Yes	No
		Leuconostocaceae	Yes	No
		Streptococcaceae	Yes	No
Clostridia	Clostridiales	Clostridiaceae	No	Yes
		Eubacteriaceae	No	No
		Heliobacteriaceae	Yes	Yes
		Lachnospiraceae	No	Yes
		Peptococcaceae	No	Yes
		Peptostreptococcaceae	Yes	Yes
		Ruminococcaceae	No	Yes
	Halanaerobiales	Halanaerobiaceae	No	No
	Thermoanaerobacteriales	Thermodesulfobiaceae	No	No
Erysipelotrichia	Erysipelotrichales	Erysiopelotrichaceae	Yes	Yes
Negativicutes	Selenomonadales	Acidaminococcaceae	No	Yes
		Veillonellaceae	No	Yes

Only families that were detected during sequencing are included above. [1-3]

Chapter 3: A *BACH2* gene variant is associated with post-operative recurrence of Crohn's disease

Abstract

Background- Crohn's disease often requires intestinal resection, which is not considered curative. Repeat surgical intervention is necessary in over half of the patients following their initial operation. Although many genetic loci are implicated in Crohn's disease, few have been associated with post-resection recurrence.

Methods- A cohort of Crohn's disease subjects who underwent intestinal resection was analyzed to determine genetic and clinical factors associated with post-resection recurrence. Genotype was assessed at eight loci associated with adaptive immunity (*SMAD3, IL10RB, IL15RA, BACH2, IL12B, IL18RAP, IFNGR2*, and *JAK2*). Univariate and multivariate survival analyses were performed using a log-rank test and Cox-proportional hazard model, respectively.

Results- 191 Crohn's disease subjects with 11.2 years mean post-operative follow-up were included. 46% experienced a surgical recurrence. Factors associated with increased incidence of recurrence included male gender($p=0.05$), and shortened time to first intestinal surgery (5.0 vs 7.3years $p=0.03$), while inflammatory disease behaviour was associated with a lower chance of repeat surgery($p<0.01$). Of the loci assessed on multivariate analysis, homozygosity for a risk allele at *BACH2* (rs1847472) was significantly associated with disease recurrence (HR-1.24 CI-1.00-1.54 $p<0.05$).

Conclusions- We identify *BACH2* as a susceptibility locus for post-operative recurrence of Crohn's disease in our cohort. *BACH2* is critical in the differentiation and function of T-cells, as a regulator of B-cell activity, and is associated with several dysregulated immunologic phenomena. Its identification as a risk locus in post-operative Crohn's disease recurrence suggests a potential role for regulatory T cells, effector T cells, humoral immunity, and immunologic memory in the development of this disease process.

Introduction

Crohn's disease (CD) is a subtype of inflammatory bowel disease, which can involve inflammation at any point in the alimentary tract (Abraham, Cho and Abraham, Clara, Cho, 2009). The natural history of CD results in over half of all those diagnosed undergoing an intestinal resection at some point (Schlussel, Steele and Alavi, 2016). Unfortunately, surgery is not curative, and disease recurs in up to 80% of patients endoscopically within one year (Rutgeerts, 2003), with over 50% of patients requiring further intestinal surgery at some point (Landsend *et al.*, 2006; Schlussel, Steele and Alavi, 2016). Specific risk factors for post-operative recurrence have been identified; these include smoking status, and disease behaviour (Buisson *et al.*, 2012), but ultimately development of post-operative CD recurrence is thought to be a due to a complex interplay between genetic, environmental, microbial, and immunologic factors.

Large genome-wide association studies have identified multiple genetic susceptibility loci for inflammatory bowel disease, and CD specifically (Barrett *et al.*, 2008; Lee *et al.*, 2017). A large proportion of identified risk loci are associated with immune modulation and the regulation of host response to biotic stimuli (Lee *et al.*, 2017). However, despite the increasing understanding of the genetic influence on CD pathogenesis, the role of genetics in the realm of post-operative CD recurrence remains unclear. Initially, NOD2 risk alleles were thought to predispose individuals to recurrence following resection, but a 2013 meta-analysis did not support this result (Büning *et al.*, 2004; Solon *et al.*, 2013). Other candidate loci thought to increase the risk of post-operative recurrence include those associated with SMAD3 and CARD8(Fowler *et al.*, 2014; Germain *et al.*, 2015).

The interaction between microbes and the human immune system appears to play a crucial role in CD development (Geremia *et al.*, 2014) and is likely to be preserved in the post-operative period given the documented relationships between microbes and post-operative recurrence (Fedorak *et al.*, 2015; Mondot *et al.*, 2015). However, the influence of genetic variants involved in immunologic memory and the adaptive immune system have not been well studied in the case of post-operative CD recurrence. We hypothesized that previously identified susceptibility loci for CD involved in these processes (Table 3.1) would be associated with an increased rate of recurrent CD following surgery.

Methods

Study subjects

The Centre of Excellence for Gastrointestinal Inflammation and Immunity Research (CEGIIR) cohort is a large prospectively collected set of IBD patients with detailed clinical information. At the time of enrollment in the database a comprehensive medical and surgical history was completed, and subjects were followed prospectively. Diagnosis of CD was established based on standard clinical, radiologic, endoscopic, and histologic criteria. Ethics approval for this study was obtained from the University of Alberta Health Research Ethics review board. All subjects provided written informed consent to be enrolled in the CEGIIR cohort. CD subjects with at least five years of disease, and an available genotype were included in the analysis. This project collected demographic information as well as relevant clinical and social information from this database.

The primary outcome measure was time to repeat CD-related intestinal resection. Perianal surgeries and operations not involving intestinal resection were excluded. Surgery for early operative complications were excluded.

Genotyping

Venous blood was obtained at the time of consent for all enrolled subjects, and stored at -80° Celsius. Genomic DNA was extracted from the samples using the Qiagen DNAeasy kit (Germantown, MD, USA). Samples were processed by BGI-Shenzhen using the Goldengate platform (Illumina). All genotyping was blinded toward clinical and demographic factors. ‘Homozygous’ genotype was defined as both loci possessing the

risk allele, ‘heterozygote’ as possessing one copy of the risk allele, and ‘wild-type’ as possessing no copies of the risk allele.

Statistical analysis

STATA 13.1 software was used to conduct all analysis. Continuous variables are presented as means (standard deviation). When continuous data met normality assumptions according to the Shapiro-Wilkes test, the Student t-test was used, otherwise the Mann-Whitney U test was performed. Categorical variables were compared using the Chi-squared test, or Fisher’s exact when any group was rare ($n<5$). All polymorphisms were tested for Hardy-Weinberg equilibrium before further analysis. Given the unknown function of studied alleles, all variants were modeled using both a dominant and recessive inheritance model. Univariate analysis of genetic loci was performed using a log-rank test, with time to surgical recurrence or to the end of follow-up used at the time variable. Kaplan-Meier curves were created using this analysis. A Cox proportional hazard model was constructed using the *a priori* selected criteria of variables reaching a p value of <0.10 in the univariate analysis and smoking status. Statistical significance was defined as $p\leq0.05$ following multivariate analysis.

Results

Patient characteristics

Of the 566 CD subjects enrolled in the CEGIIR cohort, 254 had undergone an intestinal resection for CD (45%). Of those, 191 had an available genotype (75%) (Figure 3.1). In these subjects mean age at diagnosis was 26.6 years (SD-11.2), and 42% of subjects were male. 11% subjects had purely inflammatory disease, 26% had stricturing disease, and 64% had penetrating disease based upon the Montreal classification of disease behaviour (Silverberg *et al.*, 2005). The most common disease location was ileal (47%), followed by colonic (28%) and ileo-colonic (26%). The mean length of follow-up following the initial resection was 11.2 years (SD-9.6).

Surgical recurrence

87 subjects (46%) experienced surgical recurrence within the follow-up period. Clinical characteristics and their association with recurrence are shown in Table 3.2. Univariate analysis revealed male gender (p=0.05) and time to first surgery from diagnosis (p=0.03) as increasing the likelihood of recurrence, while inflammatory disease phenotype was less likely to result in repeat surgery (p<0.01). All alleles were found to be in Hardy-Weinberg equilibrium (Table 3.3), and a genetic association was found on univariate analysis with homozygosity for a risk allele at *BACH2* (rs1847472) (Figure 3.2 p=0.04). In multivariate analysis, homozygosity at *BACH2* remained significant and independently associated with surgical recurrence (HR-1.24 CI-1.00-1.54 p<0.05) as did male gender (HR-1.91 CI-1.24-2.94 p<0.01), and inflammatory disease behaviour (HR-0.20 CI-0.06-0.63 p<0.01).

88 subjects were found to homozygote for the risk allele at rs1847472, of which 47 recurred. 83 possessed a single copy of the risk allele, while 20 did not possess any copies of the risk allele. A total of 40 of these subjects recurred. Time to recurrence was 5.7 years (SD-6.7) in the homozygote group, and 7.8 years (SD-8.9) in the remaining recurrent subjects (p=0.17).

Discussion

Knowledge of CD genetic risk loci and their effect on the risk of disease progression continues to evolve. However, there is a relative paucity of evidence regarding the role of genetics in the post-operative recurrence of CD. Here we identify a novel genetic locus, related to the *BACH2* gene, independently-associated with an increased incidence of surgical recurrence in a cohort of CD patients.

The first identified, and most well-studied genetic variants regarding CD occurs at the *NOD2* loci (Ogura *et al.*, 2001). These variants are well established as predisposing one to development of CD, and to certain disease behaviour (Limbergen *et al.*, 2009; Cleynen *et al.*, 2016). The identification of a *NOD2* risk loci, and the gene's role in bacterial peptide recognition led to increased interest in the role of microbial-immune system interactions in the development of the disease (Al Nabhani *et al.*, 2017). Similarly, understanding the genetics of post-operative CD recurrence may help to delineate the pathology of the disease.

Recent work has identified *SMAD3*, and *CARD8* as potential susceptibility loci in the development of post-operative CD (Fowler *et al.*, 2014; Germain *et al.*, 2015). Both loci are involved in major inflammatory pathways, where *SMAD3* mediates transforming growth factor-beta signaling (Flanders, 2004), and *CARD8* regulates nuclear factor-kappa B (Bouchier-Hayes *et al.*, 2001). We focused on the relatively understudied topic of adaptive immunity in the recurrence of post-operative CD, and in doing so identified *BACH2* as a potential susceptibility locus for disease recurrence following surgery. The protein encoded by *BACH2* is a highly conserved member of the basic leucine zipper-domain superfamily of transcription factors. Its importance in the maturation of B

lymphocytes has been long recognized, and its importance in T-cell differentiation has more recently come to light. A number of autoimmune diseases are associated with polymorphisms of *BACH2,* including asthma(Ferreira *et al.*, 2011), type I diabetes mellitus (Cooper *et al.*, 2008), celiac disease(Dubois *et al.*, 2010), vitiligo(Jin *et al.*, 2012), multiple sclerosis(Sawcer *et al.*, 2011), and CD(Franke *et al.*, 2010). *BACH2* has been implicated as playing a key role in immunologic tolerance. A deficiency in BACH2 leads to pulmonary inflammation, and inflammation of the alimentary tract (Roychoudhuri *et al.*, 2016), secondary to a deficiency of regulatory T cells (T_{reg}) (Roychoudhuri *et al.*, 2013; Kim *et al.*, 2014). Furthermore, BACH2 serves to lessen the induction of inflammatory cytokines IFNγ, IL-13, IL-4, IL-5 and IL-17A (Roychoudhuri *et al.*, 2013; Tsukumo *et al.*, 2013; Kuwahara *et al.*, 2014, 2016). Genomic mapping indicates that BACH2 binds enhancers associated with effector T cell differentiation (Kuwahara *et al.*, 2016; Roychoudhuri *et al.*, 2016). A mutation effecting *BACH2* therefore may shift the immunologic profile away from immunotolerance (affected by T_{reg} cells) and toward immune activation (affected by effector T cells). The identification of colitis in humans with a *BACH2* missense mutation further suggests its role in immunomodulation (Afzali *et al.*, 2017).

The role of BACH2 in restraining T cell effector programs enhances the development of immunologic memory. BACH2 is required for the differentiation of long-lived memory CD8+ T cell responses (Hu and Chen, 2013; Roychoudhuri *et al.*, 2016), and plays a role in humoral immunity as it is required for the proliferation of antigen-stimulated B cells, germinal centre B cell differentiation, class-switch recombination and somatic hypermutation (Muto *et al.*, 2004; Shinnakasu *et al.*, 2016). Given the microbial community destruction and reconstitution following intestinal resection, it stands to reason

that immunologic memory may play a crucial role in enabling appropriate microbial intestinal recolonization following surgery. Therefore, we hypothesize that a *BACH2* mutation may predispose an individual with CD undergoing intestinal resection to be at increased risk of an inappropriate inflammatory response to microorganisms responsible for healthy microbial community restoration.

The identification of inflammatory disease behaviour as being negatively associated with recurrence is consistent with prior work (Fowler *et al.*, 2014). In our cohort, male sex was independently associated with recurrence. Prior work has been split on the influence of sex, where some studies demonstrated increased risk with female gender (Atwell, Duthie and Goligher, 1965), while one prior work suggested male gender as a risk factor (O Bernell, Lapidus and Hellers, 2000). In the majority of studies gender has not demonstrated an influence on disease recurrence (De Cruz *et al.*, 2012).

Genetic studies of this type are biased toward false discovery, and though *BACH2* is an independent risk locus for recurrence in our cohort, replication in other groups must be sought before definitively labelling rs1847472 as a risk locus. Further, the HR for *BACH2* homozygosity was modest, and its role in diagnosis and prediction of post-operative recurrence would be limited. Nonetheless, its identification as a risk locus may enable mechanistic understanding of the pathogenesis of post-operative recurrence. Finally, the nature of data collection in this study did not enable collection of important potentially confounding data including pre and post-operative medication usage, and peri-operative complications.

The identification of rs1847472, associated with the *BACH2* gene, as an independently-associated risk locus in the development of post-operative recurrence of CD following

intestinal resection opens interesting avenues of investigation in the pathogenesis of recurrence. Should this association be confirmed in separate cohorts, the role of T_{reg} cells, effector T cells, humoral immunity, and immunologic memory must be considered as important potential mechanisms in the development of this disease process.

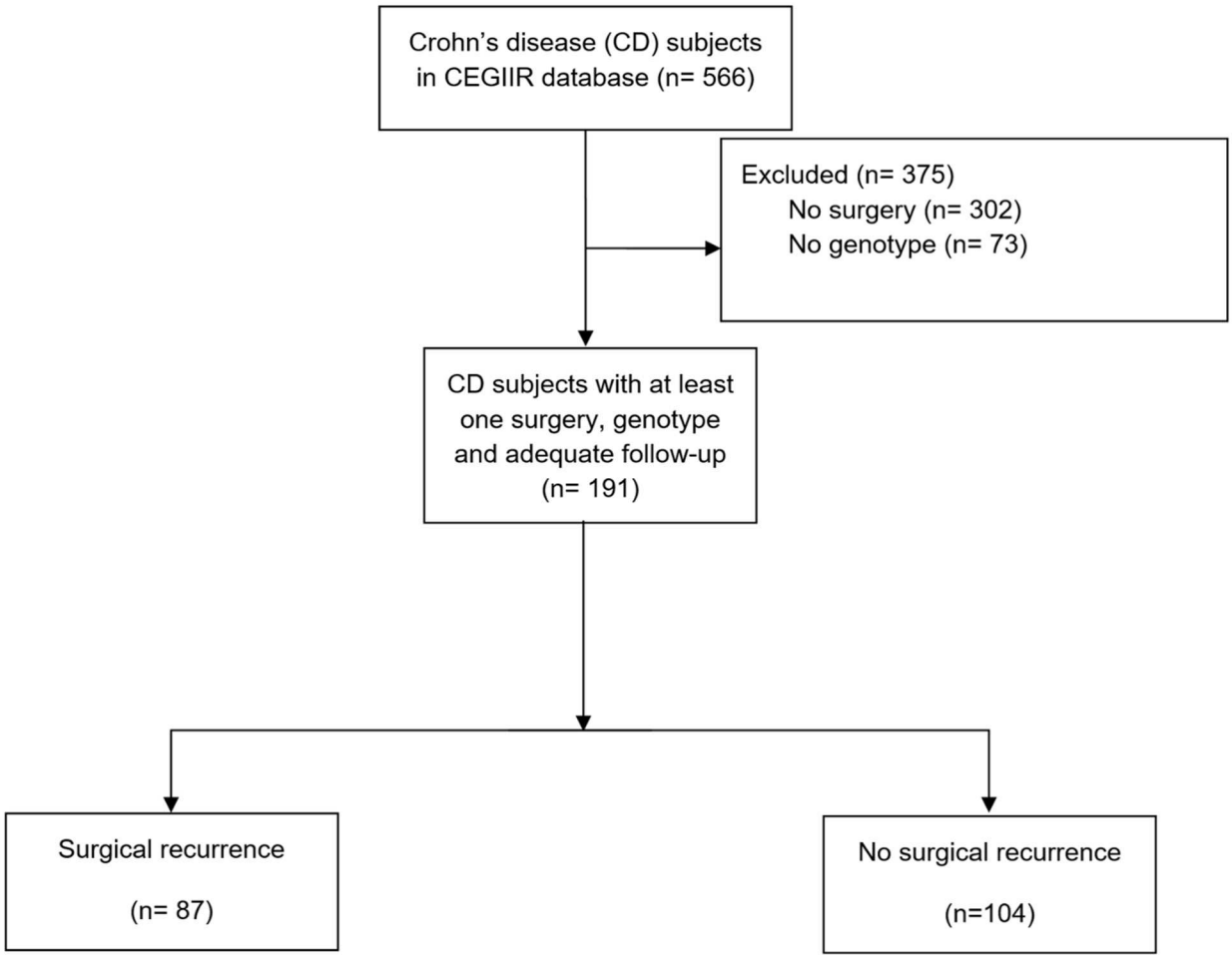


Figure 3.1: Summary of Crohn's disease patients from the CEGIIR cohort included in the analysis

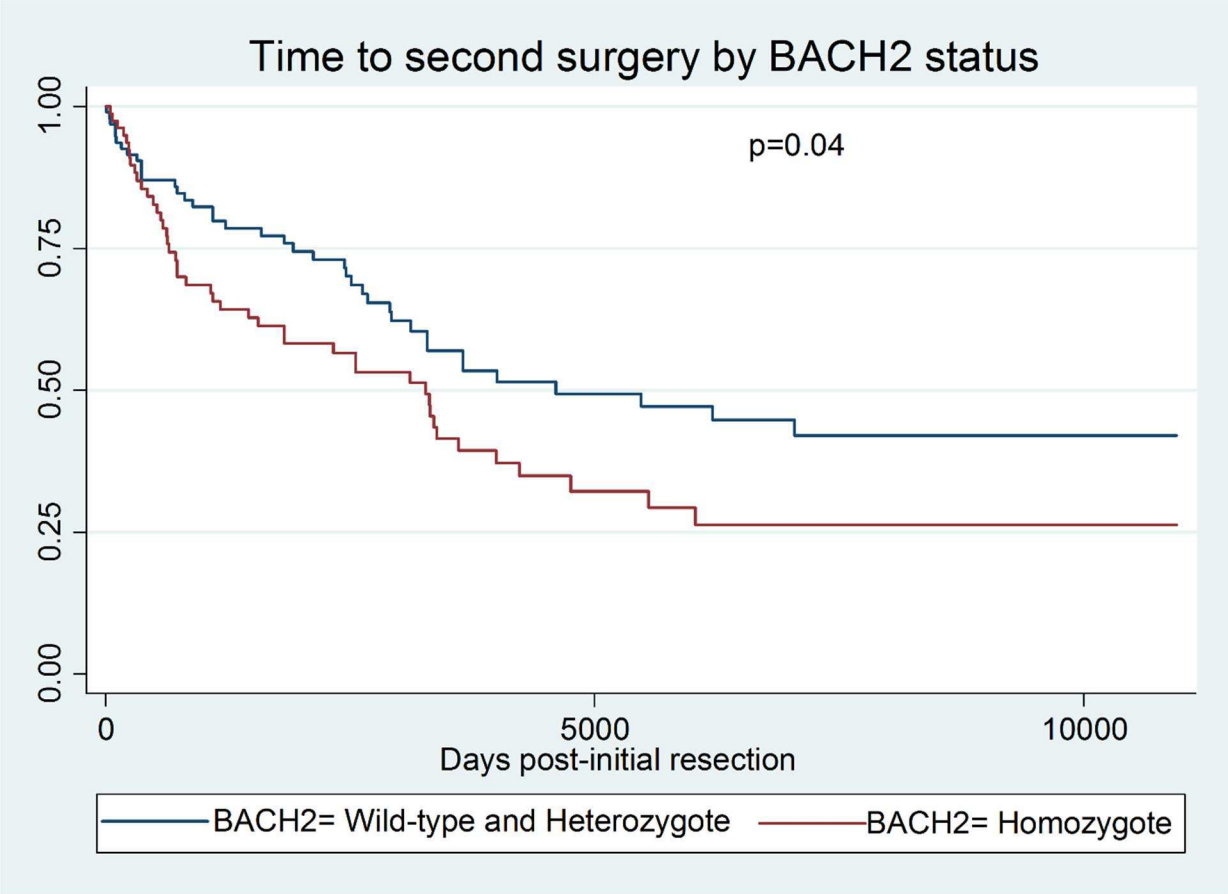


Figure 3.2: Survival curve for time to surgical recurrence according to *BACH2* status. P-value calculated using the log-rank test.

BACH2 homozygotes (n=88) *BACH2* heterozygotes and wild-type (n=103)

Table 3.1: Summary of risk loci for CD examined in this study

Gene(s)	Single nucleotide polymorphism (SNP)	Role in adaptive immunity
SMAD3	rs17293632	Key molecule in TGF-β signalling, which acts as a regulator of effector and regulatory CD4(+) T cell responses(Flanders, 2004; Travis and Sheppard, 2014)
IL10RB	rs2284553	A subunit for the interleukin-10 receptor. Interleukin-10 plays a key role in adaptive immunity, by mediating immunosuppression via Treg cell (Ng *et al.*, 2013)
IL15RA	rs12722515	A subunit for the interleukin-15 receptor. Interleukin-15 has broad effects in both adaptive and innate immunity (Lodolce *et al.*, 2002; Inoue *et al.*, 2010)
BACH2	rs1847472	A key molecule in regulating T cell differentiation, immunologic memory, and humoral immunity (Igarashi, Kurosaki and Roychoudhuri, 2017)
IL12B	rs6871626	A subunit of interleukin-12, which is induced by microbial products and can promote T cell expansion and proliferation (Watford *et al.*, 2003)
IL18RAP, IL18R1	rs917997	Genes important for interleukin-18 receptors, which facilitate activation of T-cells (Dinarello *et al.*, 2013)
IFNGR2	rs2284553	A signalling receptor that plays a role in shaping T-helper subsets (Holzer *et al.*, 2013)
JAK2	rs10758669	A signalling molecule involved in the differentiation of T helper cells (Limbergen *et al.*, 2009)

Table 3.2: Clinical characteristics of CD patients by surgical recurrence

	No recurrence (n=104)	**Recurrence (n=87)**	**p-value**
Male gender (%)[a]	36%	49%	**0.05**
Total follow-up time (mean years (SD))	17.5 (10.5)	16.6 (8.3)	0.52
Time to first surgery from diagnosis (mean years (SD))[a]	7.3 (7.9)	5.0 (6.0)	**0.03**
Follow-up following first operation (mean years (SD))	10.5 (11.0)	12.0 (7.7)	0.28
Disease Location			
Ileal (%)	43%	51%	0.65
Colonic (%)	28%	27%	0.66
Ileocolonic (%)	28%	22%	0.29
Disease Behaviour			
Inflammatory (%)[a]	17%	3%	**<0.01**
Stricturing (%)	23%	29%	0.29
Penetrating (%)	60%	67%	0.26
Age of CD diagnosis (mean years (SD))	26.8 (11.4)	24.6 (12.8)	0.21
Peri-anal disease (%)	35%	39%	0.56
Smoking history (%)	59%	59%	0.98

a- Variables included in the multivariate analysis

Table 3.3: Population distribution of the studied alleles and association with recurrence on univariate analysis

Gene(s)	SNP	HW p-value	Risk allele frequency (Recurrence)	Risk allele frequency (remission)	Dominant inheritance p-value*	Recessive inheritance p-value*
SMAD3	rs17293632	0.11	0.30	0.28	0.44	0.21
IL10RB	rs2284553	0.44	0.68	0.63	0.71	0.43
IL15RA	rs12722515	0.14	0.85	0.85	0.90	0.56
BACH2	rs1847472	0.95	0.69	0.66	0.40	0.04
IL12B	rs6871626	0.99	0.37	0.40	0.61	0.63
IL18RAP, IL18R1	rs917997	0.95	0.28	0.25	0.69	0.14
IFNGR2	rs2284553	0.44	0.68	0.63	0.71	0.43
JAK2	rs10758669	0.71	0.40	0.42	0.54	0.44

SNP- Single nucleotide polymorphism

HW p-value- Test for deviation from Hardy-Weinberg equilibrium

* univariate p-values calculated using the Log-rank test

Chapter 4- Cytokines for the prediction of recurrent Crohn's disease following intestinal resection

Introduction

Crohn's disease (CD) is defined by inappropriate inflammation occurring anywhere within the alimentary tract (Abraham, Cho and Abraham, Clara, Cho, 2009). The pathophysiology of CD is not completely understood, but a combination of immunologic, genetic, microbial, and environmental causes seems to play a role in disease development. Cytokines, small proteins important in cellular signaling, play a central role in the inflammatory process seen in CD, and perturbations between pro-inflammatory and anti-inflammatory cytokines has important implications on the disease state (Neurath, 2014).

Progression of CD leads to intestinal resection in approximately half of all those individuals diagnosed (Schlussel, Steele and Alavi, 2016). Surgery is not curative, and disease recurs within one year in up to 80% of patients (Rutgeerts, 2003). The pathophysiology of recurrence is poorly understood, and it is unknown which immunologic pathways in the post-operative period lead to recurrent inflammation. Recent work has examined the role of microbial and genetic factors present at the time of surgery that predicts subsequent recurrence but relatively little work has focused on inflammatory molecules (i.e. cytokines) present at the time of resection and their relationship to recurrence (Fowler *et al.*, 2014; Germain *et al.*, 2015; Mondot *et al.*, 2015). Cytokine signalling post-operatively in CD varies temporally and by disease state (Zorzi *et al.*, 2013), though recurrence seems to be foreshadowed by differential expression of major immune signaling molecules, including interleukin (IL)-10 and TGF-β (Meresse *et al.*, 2002; Scarpa *et al.*, 2009). A number of cytokines have been implicated in the development of CD, including IFNγ, TNF, IL-6, IL-10, IL-12, IL-12 and IL17A (Neurath, 2014). We hypothesized that inflammatory signaling molecules in the mucosa and mesenteric lymph nodes at the time of surgery would be predictive of future recurrent endoscopic disease; further, specific cytokines/chemokine may be identified that would

be involved in the critical inflammatory pathways responsible for intestinal inflammation in post-operative recurrence of CD.

Methods

Patient Cohort

Twenty-six subjects were recruited between June 2012 and June 2014 at the University of Alberta Hospital, Royal Alexandra Hospital, Grey Nuns Hospital, or Misericordia Hospital at the time of intestinal resection for CD. Approximately six months following surgery endoscopic remission was assessed by the treating gastroenterologist using the validated Rutgeerts score with recurrence being defined as ≥ 2. Demographic and clinical information was obtained from the subject's medical record at the time of surgery and follow-up endoscopy. Post-operative therapy was not standardized and was determined by the subject's clinical gastroenterologist. This study was approved by the Health Research ethics board at the University of Alberta (Pro00028147).

Sample collection

Biopsies were taken from the mucosa of the terminal ileum in macroscopically normal tissue (n=26), and in mesenteric lymph nodes of the surgical specimens (n=15). All samples were snap frozen immediately after collection in liquid nitrogen and stored at -80ºC. Biopsies were homogenized in PBS with 0.05% Tween 20. These homogenates were then centrifuged at 10 000 rpm for ten minutes. The supernatant was retained and applied to a Meso Scale discovery system (Meso Scale Diagnostics, Gaithersburg, MD, USA) as per the manufacturer's protocol.

Statistical analysis

Analysis was performed using STATA (StataCorp. 2013. College Station, TX) and Metaboanalyst (Xia *et al.*, 2015). Cytokine tissue concentrations were log-transformed and compared using the Student t-test when data fit a normal distribution and Mann-Whitney U test otherwise.

Results

Demographic information regarding the cohort can be found in Table 4.1. Of the 26 analyzed subjects, 31% recurred. Those who recurred tended to be older (48.0 (8.3) vs. 38.5 (10.9) $p=0.03$). No other statistically significant differences were found between those who recurred or relapsed in terms of the available clinical characteristics ($p>0.05$). No subjects were found to have severe disease recurrence (as defined by Rutgeerts score of 4) at follow-up.

Detailed cytokine data from each subject is available in Figure 4.1. Cytokine concentrations from samples were analyzed based upon disease behaviour, and disease recurrence. Disease recurrence was found to be associated with an elevation in CCL2 in the ileal mucosa of each surgical specimen ($p<0.01$ Figure 4.2a). A depression in the levels of IL-5 ($p<0.01$ Figure 4.2b) and IL-16 ($p<0.01$ Figure 4.2c) in the mesenteric lymph nodes sampled at the time of surgery were associated with disease recurrence. Elevated IL-6 was associated with a stricturing disease phenotype ($p<0.01$ Figure 4.2d).

Discussion

The role of cytokines, and specifically the balance between pro and anti-inflammatory signalling molecules appears to be central in the development and perpetuation of inflammatory bowel disease(Neurath, 2014). The cytokines implicated in disease evolution regulate a vast network of signalling pathways and are derived from a variety of immune cell types. Tumour necrosis factor (TNF)-α is one such molecule, whose presence promotes hypervascularization, angiogenesis, and pro-inflammatory cytokine release in the IBD patient (Di Sabatino *et al.*, 2007; Meijer *et al.*, 2007; Atreya *et al.*, 2011; Günther *et al.*, 2011; Su *et al.*, 2013). The understanding of TNF-α as central inflammatory signalling molecule in IBD led to the development of antibodies that target and neutralize the molecule (Atreya *et al.*, 2011). The development of TNF-α as a therapeutic target highlights the potential clinical value of identifying specific signalling pathways in inflammatory disease pathogenesis.

Previous investigations into the inflammatory signalling pathways in the post-operative recurrence of CD have revealed a potential role for TGF-β1 and IL-6 in the intestinal mucosa (Scarpa *et al.*, 2009; Ruffolo *et al.*, 2010). Both of these molecules have been shown to induce the production of CCL2, the mucosal cytokine associated with disease recurrence in our cohort of subjects (Matagrano, Magida and McGee, 2003; Caiello *et al.*, 2014; Lerrer *et al.*, 2017). This molecule is central in the regulation of macrophage and monocyte balance, a balance which appears to be critical following intestinal resection and in CD generally (Schwarzmaier *et al.*, 2013; Bain and Mowat, 2014; Gren and Grip, 2016; Perry *et al.*, 2017). Macrophages play a central role in the interface between microbes and their human host, a relationship that is thought to be perturbed in CD. Aberrant over-expression of CCL2 in the gut of a surgical CD patient may therefore lead to improper restoration of microbial-host interactions, and subsequent perpetuation of inflammation. A relationship between IL-6 and stricturing disease was noted in our cohort in keeping with IL-6's known role in fibroblast activation, cellular proliferation and extracellular matrix production, which may be secondary to abnormal STAT3 phosphorylation (Li and Kuemmerle, 2014; C. Li *et al.*, 2015).

The majority of work examining inflammatory molecule signalling in inflammatory bowel disease has focused on local (i.e. intestinal mucosa) or systemic (i.e. serologic) observations, largely due to the relative ease of sampling these areas. The role of regional immune response is less well understood, despite evidence that mesenteric lymph nodes are a key pathogenic location in CD and site of a unique inflammatory cytokine milieu (Sakuraba *et al.*, 2009). The surgical subject offers the opportunity to focus on the importance of this regional response. Reduced concentrations of two cytokines in the MLN were associated with disease recurrence, namely IL-16 and IL-5. The role of IL-16 as a negative predictor of post-operative recurrence may lay in its property as a T-cell chemoattractant. IL-16 preferentially recruits T-regulatory cells, a group of immune cells typically thought to be immunotolerant (McFadden *et al.*, 2007). Therefore, decreased expression of this protein following surgery may shift the balance of T-cell subsets toward inflammation in the regional lymph node basin of post-operative CD patients. IL-5 has previously been shown to be elevated in ulcerative colitis patients, but not CD patients (Műzes *et al.*, 2012; Davoine and Lacy, 2014). Given IL-5's critical role in B-cell maturation (Horikawa and Takatsu, 2006), a lack of IL-5 in those who recur suggest that B-cell function, and potentially humoral immunity, may be central in prevention of post-operative recurrence of Crohn's disease.

Care must be taken in interpreting the presented data, as this work is intended as an exploratory, hypothesis-generating study, and correction for multiple testing was not performed. Further, this study does not provide mechanistic insight into the role of each cytokine and their respective association to clinical outcomes.

Ultimately, this work acts as a hypothesis generating experiment, identifying CCL2, IL-16 and IL-5, and their associated signalling pathways, as potentially important in the development or prevention of post-operative recurrence of CD.

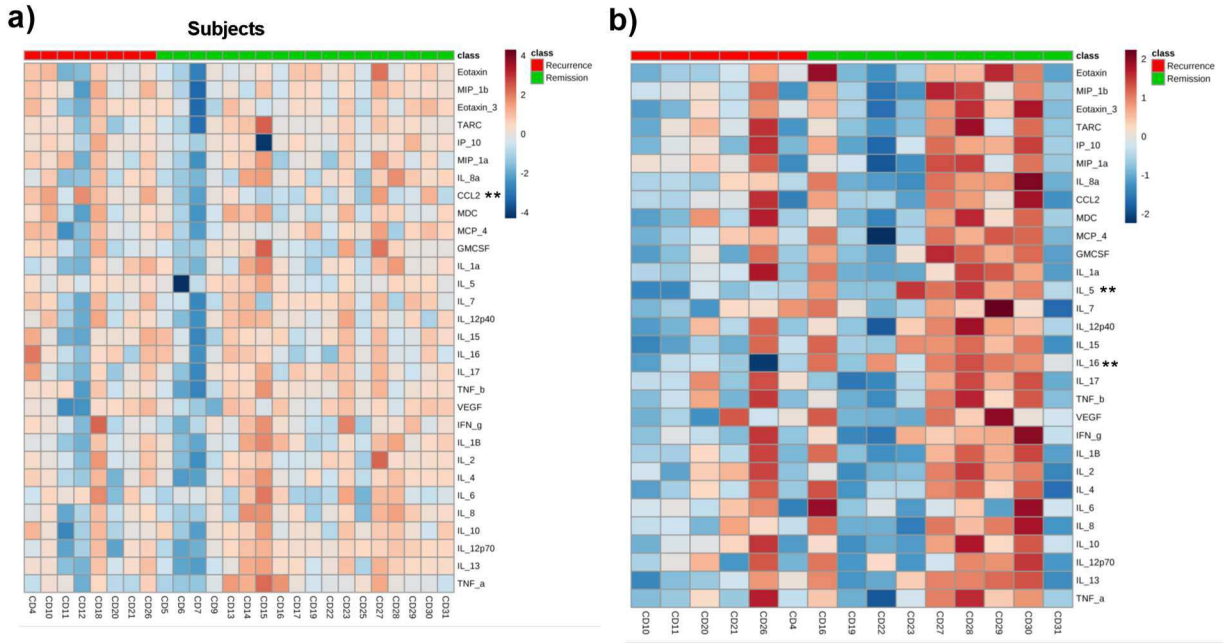


Figure 4.1: Relative cytokine expression in the ileal mucosa and mesenteric lymph nodes of subjects with Crohn's disease undergoing intestinal resection.

a) A heatmap of relative cytokine expression in the ileal mucosa of individual surgical specimens, grouped by recurrence (left) and remission (right). CCL2 is expressed at a higher level in those individuals who ultimately recur (** $p<0.01$).

b) A heatmap of relative cytokine expression in the mesenteric lymph nodes of individual surgical specimens, grouped by recurrence (left) and remission (right). IL-5 and IL-16 are expressed at decreased levels in subjects who ultimately recur (** $p<0.01$).

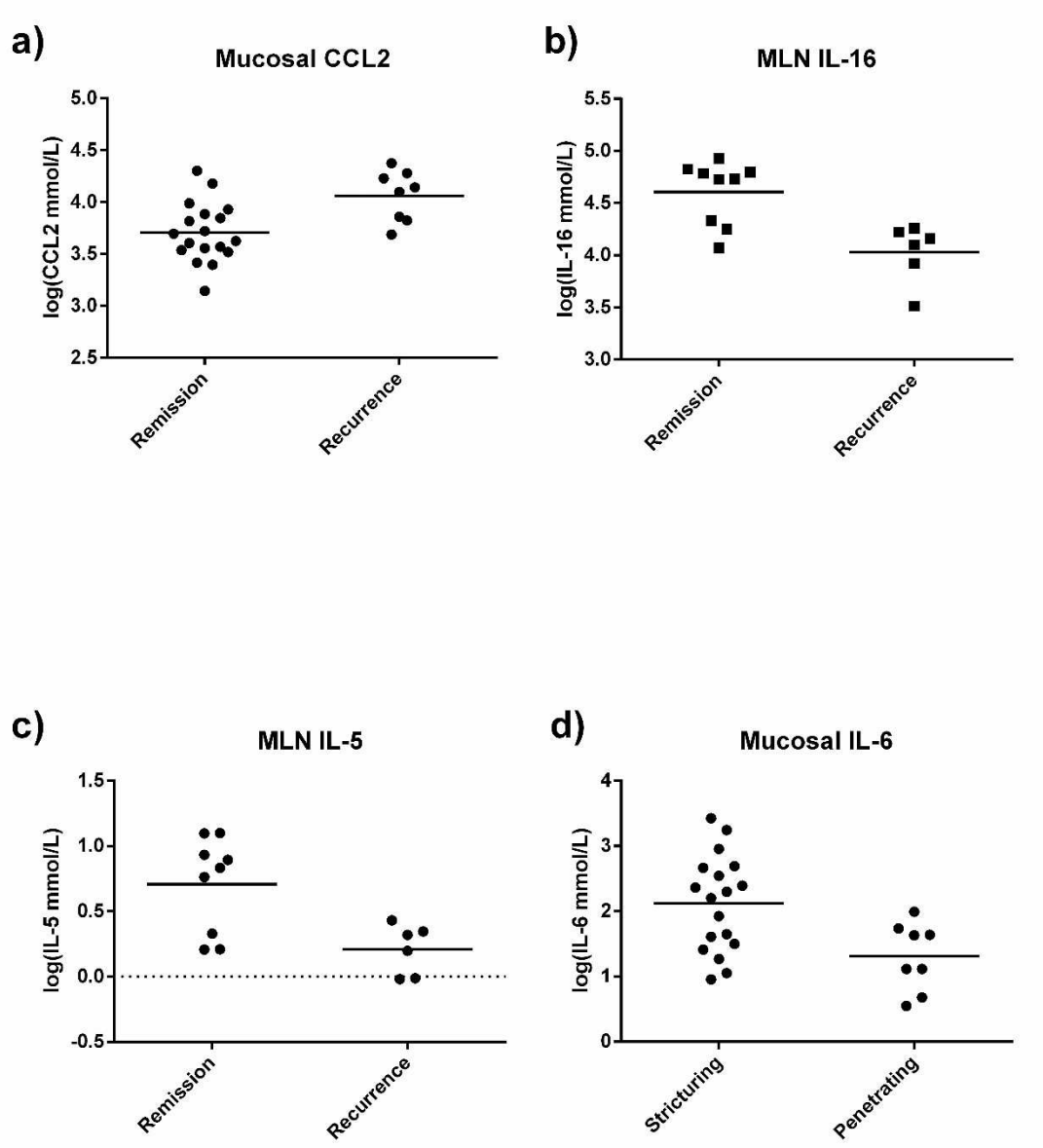


Figure 4.2: The cytokines CCL, IL-5, and IL-16 are associated with disease progression, while elevated IL-6 is associated with a stricturing disease phenotype.

a) The mean concentration of CCL2 in the ileal mucosa of subjects who recurred was greater than in those who remained in remission ($p<0.01$)
b) The mean concentration of IL-5 in the mesenteric lymph nodes of subjects who recurred was less than in those who remained in remission ($p<0.01$)
c) The mean concentration of IL-16 in the mesenteric lymph nodes of subjects who recurred was less than in those who remained in remission ($p<0.01$)
d) The mean concentration of IL-6 in the ileal mucosa of subjects manifesting a stricturing phenotype was greater than in those who with a penetrating disease behaviour($p<0.01$)

Table 4.1: Clinical and demographic characteristics of subjects by recurrence status

	Recurrence (n=8)	**Remission** (n=18)	**P**
AGE AT OPERATION(YEARS (SD))	50.0 (4.0)	38.0 (11.1)	**0.03**
MALE (%)	25%	33%	NS
PREVIOUS ICR (%)	25%	56%	NS
AVERAGE TIME TO ENDOSCOPY (DAYS)	176 (52)	209 (117)	NS
CURRENT SMOKER (%)	0%	17%	NS
PERI-ANAL DISEASE (%)	0%	17%	NS
DISEASE BEHAVIOUR			
STRICTURING (%)	75%	67%	NS
PENETRATING (%)	25%	33%	NS

Chapter 5: Prebiotic supplementation following ileocecal resection in a murine model is associated with a loss of microbial diversity and increased inflammation

Abstract

Background: Individuals with Crohn's disease frequently require ileocecal resection (ICR), and inflammation often recurs in the neo-terminal ileum following surgery. Fructooligosaccaride (FOS) is a fermentable prebiotic that stimulates the growth of *Bifidobacterium* and may promote anti-inflammatory activity. The aim of this study was to determine if supplementation of a post-ICR diet with FOS in a mouse model would be effective in stimulating the growth of bifidobacteria and reducing systemic and local inflammation.

Methods: ICR was performed in IL10-/- mice (129S1/SvlmJ) with colitis. Following surgery, non-ICR control and ICR mice were fed a chow diet ± 10% FOS for 28 days. Serum, colon, and terminal ileum (TI) were analyzed for cytokine expression by MesoScale discovery platform. DNA extracted from stool was analyzed using 16s rRNA sequencing and qPCR. Expression of *occludin* and *ZO1* was assessed using qPCR. Short-chain fatty acid (SCFA) concentrations were assessed using gas chromatography.

Results: ICR led to increased systemic inflammation ($p<0.05$) and a significant decline in fecal microbial diversity ($p<0.05$). Mice on the FOS diet had a greater reduction in microbial diversity and also had worsened inflammation as evidenced by increased serum IL-6 ($p<0.05$), ileal IL-1β ($p<0.1$) and colonic IFNγ and TNFα ($p<0.05$). Expression of occludin and ZO1 were significantly reduced in FOS-supplemented mice. There was a correlation between loss of diversity and the bifidogenic effectiveness of FOS ($r= -0.61$, $p<0.05$).

Conclusions: FOS-supplementation of a post-ICR diet resulted in a decrease in fecal bacterial diversity, reduction in barrier function, and increased gut inflammation.

Introduction

Mammals have evolved to house diverse microbial communities at many sites throughout the body, which has resulted in an intimate relationship between microbes and their mammalian host (Ley, Lozupone, *et al.*, 2008). These relationships are at the forefront of intestinal health and disease (Bindels *et al.*, 2015). Abnormal microbial composition, or microbial dysbiosis, has been described in numerous autoimmune and chronic human diseases (Packey and Sartor, 2009; Bindels *et al.*, 2015). Crohn's disease, a subtype of inflammatory bowel disease, has been extensively studied in this regard, and although there appears to be a relationship between host inflammation and microbial dysbiosis, the extent, importance and nature of that dysbiosis is unclear (Packey and Sartor, 2009; Gevers *et al.*, 2014). This relationship is further complicated by the fact that microbes can produce active metabolites that interact with the intestinal mucosa, or influence the activity of other microorganisms. One such interaction is cross feeding, whereby the end product of a microbe's fermentation process serves as a substrate for another microbe's metabolic processes (Vuyst and Leroy, 2011; Rios-Covian and Gueimonde, 2015). Intestinal microbial diversity decreases in the presence of active inflammatory processes, with certain bacterial communities, such as Proteobacteria, thriving under inflammatory conditions and other strict anaerobes, such as bifidobacteria, disappearing (Sha *et al.*, 2013). A loss of these anaerobic species also removes many physiological active metabolites that have anti-inflammatory and immune regulatory actions, thus potentially exacerbating inflammation.

Many patients with Crohn's disease require an intestinal resection at some point, with the most common resection consisting of removal of the final portion of the small intestine

and first portion of colon, called ileocecal resection (ICR) (Peyrin-Biroulet, Loftus, *et al.*, 2009). The post-operative Crohn's disease gut offers an interesting opportunity to examine the relationship between microbes and inflammation. Surgery aims to remove all actively diseased tissue, thus providing a proverbial inflammatory clean slate. In addition, ICR also will remove a large proportion of bacteria at the diseased site, and presumably leaves the patient with a microbial clean slate. However, despite this, it has been shown that anywhere from 30-90% of patients suffer from endoscopic recurrence of disease at the site of their anastomoses within one year (Rutgeerts, 2003; Lee *et al.*, 2014; Onali *et al.*, 2016). It is possible that this recurrence is driven by a persistently dysbiotic microbiota left after surgery that is perpetuated and initiates disease recurrence. This concept is supported by the findings that probiotic use initiated immediately after surgery has a beneficial effect at early time points (Fedorak *et al.*, 2015).

The addition of prebiotics to the diet is a method of targeting specific microbes to increase their abundance. Fructo-oligosaccaride (FOS) is a prebiotic that can be utilized by bifidobacteria and has been shown to increase relative abudance of both *Lactobacillus* and *Bifidobacterium* in the gut (Fishbein, Kaplan and Gough, 1988; Kaplan and Hutkins, 2000). Given the lack of bifidobacteria in the inflammatory disease state (Sha *et al.*, 2013) and the ability of bifidobacteria to produce and enable the production of putatively anti-inflammatory metabolites such as acetate and butyrate (Vuyst and Leroy, 2011; Rios-Covian and Gueimonde, 2015), we hypothesized that in a murine model of post-ICR Crohn's disease, the addition of FOS would increase the relative abundance of bifidobacteria and subsequently decrease local and systemic inflammation following surgery.

Methods

Animal Model

The animal use protocols employed were approved by the animal care committee at the University of Alberta. IL-10 -/- (129 Sv/Ev) mice underwent ICR as previously described (Perry *et al.*, 2014). The terminal ileum was transected proximal to the cecum and the descending colon was divided at the distal cecum. The anastomosis was created using 8-0 Prolene (Ethicon) sutures in a simple interrupted fashion and the abdominal wall was closed. Mice who had undergone ICR were maintained on liquid diet for two days pre-operatively and two days post-operatively. Non-operative control mice received 4 days of liquid diet but did not undergo surgery. Following the completion of liquid diet, mice were started on either the control diet (Control group (n=5) and ICR-C group (n=5)) or the FOS-supplemented diet (FOS group (n=6) and ICR-FOS group (n=6)) (Figure 5.1). Experimental diets contained a standard mouse chow, LabDiet 5001 (LabDiet, USA) (29% protein, 55% carbohydrates, 13% fat; 3.8 kcal/g; PMI Nutrition International, Richmond, IN, USA) supplemented with the addition of a dietary fibre. Control diet contained additional cellulose to 10% by dry weight. FOS-supplemented diet contained 10% FOS by dry weight.

All mice were 12 weeks of age at the initiation of the experiment. Overall survival was 92% with two mice dying post-operatively prior to the introduction of either the control or experimental diet. Animals were weighed on days 0, 14, 21 and 28 of the experiment. Food was weighed and replaced on days 0, 3, 7, 10, 14, 17, 21, 24 and 28. Stools were collected prior to the initiation of liquid diet, and on days 14 and 28. Animals were housed two per cage and littermates were randomized across treatment groups.

Histological analyses

Sections of peri-anastomotic ileum and colon were taken at day 28 and fixed in 10% buffered formalin, embedded in paraffin and cut to 5 μm. These sections were stained with either Masson trichome or hematoxylin/eosin (H&E). Histologic injury was scored by a pathologist blinded to the treatment groups using a validated ten point scale that includes enterocyte injury, epithelial hyperplasia, lamina propria lymphocytes, and lamina propria neutrophils. Collagen deposition was scored from 0-2 using the Masson trichome stains as previously described (Borowiec *et al.*, 2012).

Gut Microbial Composition

Genomic DNA was extracted from stool samples using FastDNA Spin Kit for Feces (MP Biomedicals, Lachine, QC, Canada) and quantified using PicoGreen DNA quantification kit (Invitrogen, Carlsbad, CA, USA). Sequencing was performed the MiSeq Illumina platform. Genomic DNA was subjected to a NaCl and ethanol precipitation procedure to remove contaminants that interfere with PCR. The protocol involved the addition of 5M NaCl to a total 5% of the DNA sample and precipitation of the DNA with one sample volume of ice-cold anhydrous ethanol. The samples were left at -20C for 30 minutes then centrifuged at 10,000g for 15 minutes. The liquid was discarded and one volume of ice-cold 75% ethanol was added to the pellet as a wash. The samples were centrifuged, decanted and left to dry at room temperature for 30 minutes. The DNA pellet was solubilized in EB buffer (Qiagen, USA). Microbial composition was assessed using Illumina's established 16S rRNA amplicon sequencing method and the MiSeq sequencing platform. No deviations from the manufacturer's protocol were used. Briefly, a segment of the V3 and V4 region of the 16S gene was amplified with gene specific primers (aligning

to 341bp and 805bp in the gene) that also include an adapter sequence overhang: Bact_16s_ILL1_341mF 5-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3, Bact_16s_ILL1_805mR 5- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3. This PCR reaction was cycled 25 times and the resulting reaction was purified using bead-based clean-up followed by an 8 cycle PCR reaction using Illumina's proprietary bar-coding primers that also align to the adapter sequence. After a second clean-up the bar-coded libraries were diluted, denatured, pooled and run using a V3 300bp reagent cartridge on the MiSeq system.

Bacterial composition was estimated from the data using Quantitative Insights into Microbial Ecology (QIIME 1.9.1) pipelines (Caporaso *et al.*, 2010). In brief, QIIME was used to analyze for phylogenetic and operational taxonomic unit (OTU). First of all, it was used to de-multiplex the barcoded reads and perform chimera filtering. Filtered sequence reads were grouped into OTUs at a sequence similarity level of 97%, which approximates species-level phylotypes. Taxonomy of the OTUs was assigned and sequences were aligned with RDP classifier and Pynast. To evaluate the alpha diversities of each microbiota community, we calculated the Shannon diversity metric.

16s rRNA qPCR

Genomic DNA was extracted from stools using a FastDNA Spin Kit (MP Biomedicals). The manufacturer's protocol was followed, and the resultant DNA was quantified using a PicoGreen assay (Invitrogen). The samples were then diluted to 50ng/ml and re-quantified with PicoGreen. Reactions containing 6ul of H20, 10ul of Fast SYBR Green Master Mix, and 1ul of 10uM forward and reverse primer were added to 2ul of target DNA

for quantitative PCR (qPCR) in MicroAmp 96 well optical plates. PCR conditions in the 7900HT instrument were 5 minutes at 50 degrees C, 5 minutes at 95 degrees C, 15 seconds at 95 degrees C and 1 minute at 60 degrees C, with a melting curve step progressing between 60 to 95 degrees C in 12 minutes. DNA copy number was determined by comparison to standard curves constructed from purified PCR product quantified using PicoGreen. Gene copy per nanogram of genomic DNA was determined in relation to the starting concentration of genomic DNA in each reaction.

RNA extraction and qPCR

Total RNA was isolated and extracted from mouse terminal ileum tissue. Tissues were snap frozen, then homogenized and extracted in Trizol according to the manufacture's protocol (Invitrogen Corporation, Carlsbad, CA, USA). An RNeasy mini kit (Qiagen, Inc., Mississauga, ON, USA) was used for purification, and purity was assessed using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Complimentary DNA was synthesized by the the High Capacity cDNA Reverse Transcription Kit (Invitrogen). Quantitative real-time polymerase chain reaction (qPCR) was performed using Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) for 18s rRNA, Occludin and ZO1. Each sample was run in triplicate using the 7900HT Fast Real-Time PCR System and Sequence Detection System v2.4 software (Applied Biosystem) and the relative quantification was calculated using the ΔΔCt method and compared to expression of 18S rRNA.

Assessment of serum lipopolysaccharide (LPS)

A HEK-blue cell system (InvivoGen) was used to analyze the serum concentration of LPS. The cells were cultured in a 96-well plate and 5μL of serum diluated 1:10 was

incubated with the cells for 6 hours. LPS activation of the system catalyzes the detection medium to turn blue. Activation was quantified by measuring absorption at 650 nm for quantification.

Measurement of cytokines

Segments of peri-anastomotic colon and ileum were snap frozen at collection. They were then homogenized in 7.5X tissue weight in PBS and 0.05% Tween 20. They were centrifuged at 10 000 rpm for 10 minutes and the resultant supernatant was assessed for cytokine expression. Cytokine values are corrected for tissue weight. INF-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL12p70 and TNF-α were assessed using the Meso Scale discovery system (Meso Scale Diagnostics, Gaithersburg, MD, USA) as per the manufactures protocol.

Measurements of short-chain fatty acids (SCFA)

The concentrations of SCFA in feces was determined using gas chromatography. 0.2g of feces was homogenized in 800uL of 0.1N hydrochloric acid then 200µl of 25% phosphoric acid was added. This was centrifiuged at 3 000g for ten minutes and the supernatant was added to internal standard solution (150 mg of 4-methyl-valeric acid, S381810, Sigma-Aldrich) and 5% phosphoric acid in a glass chromatography tube, mixed well, and kept at room temperature for 30 min. The supernatant was analyzed for SCFA (i.e., acetic, propionic, butyric, and isobutyric) using a Varian model 3400 Gas Chromatograph (Varian, Walnut Creek, CA) with a Stabilwax-DA column (30-m × 0.25-mm i.d.; Restek, Bellefonte, PA). A flame-ionization detector was used with an injector temperature of 170°C and a detector temperature of 190°C.

Statistical analysis

All data is presented as the mean ± the standard error of the mean. Statistical analysis was performed using STATA v13.1. Student's t-test was used when comparing two groups. Two-way analysis of variance (ANOVA) was used to compare the significance when the model consisted of both diet and ICR. Principal component analysis (PCA) plots of bacterial populations and heatmaps of cytokine expression were created using Metaboanalyst 3.0 after logarithmic transformation of the data.

Results

Effect of surgery

The surgical insult associated with ICR led to a mean weight loss of 5.2 ± 0.3% in all surgical mice at one week (Figure 5.2a). At day 28 at the time of sacrifice, mice who had undergone ICR had a significantly lower weight than at day 0 ($p<0.01$) (Figure 5.2a) and also as compared to their non-operative counterparts ($p<0.01$) (Figure 5.2b). There was a delay in the restoration of weight in ICR-FOS mice compared to ICR-C mice ($p=0.01$) (Figure 5.2a). All ICR mice consumed significantly less food during the first week ($p<0.01$), but food consumption was similar across all groups in subsequent weeks (Figure 5.2c).

Effect of surgery and FOS on inflammation and fibrosis

Post-ICR mice demonstrated increased levels of serum inflammatory cytokines, evidenced by increased IL-2, IL-12, IL-4 ($p<0.05$) (Figure 5.3a). Serum IL-6 was significantly elevated in ICR-FOS mice compared to ICR-C mice ($p=0.02$) (Figure 5.3a). Although FOS-supplementation increased colonic IFNγ ($p=0.05$) and TNFα ($p=0.04$) (Figure 5.3a), there were no significant differences in histological scores in the colon between the groups (Figure 5.3b). ICR-FOS mice demonstrated a non-significant increase in fibrosis scores compared to ICR-C mice (1.17 versus 0.6 $p=0.28$) (Figure 5.3c). ICR was associated with elevated IL-12 in the terminal ileum (Figure 5.3a).

Barrier Function

FOS-supplementation led to increased levels of serum LPS compared to control diet ($p=0.05$) (Figure 5.4a), suggesting a loss of barrier function. A loss of barrier function was also supported by the findings of a decreased expression in RNA of the tight junction proteins, occludin and ZO1 secondary to FOS-supplementation ($p=0.05$ and $p=0.04$

respectively) and ICR ($p<0.01$ and $p=0.04$ respectively) (Figure 5.4b and c).

Microbial Composition

The baseline microbiota across all groups was similar (Figures 5.5a and c). qPCR analysis revealed a large drop in the total number of fecal bacteria following surgery ($p<0.01$) (Figure 4e). FOS-supplementation was associated with a further decrease in levels of fecal bacteria ($p=0.04$). As expected, although there was decreased *Bifidobacterium* post-ICR in all surgical mice, FOS-supplementation did result in an increase in *Bifidobacterium* in both controls and ICR mice, albeit to a much decreased level in the post-ICR mice ($p<0.0001$) (Figure 5.5f).

ICR was responsible for a precipitous decrease in bacterial diversity as measured by the Shannon index (Control diet: 7.39±0.30 v ICR-C: 2.77±0.37 $p=0.01$) and in richness as evidenced by the rarefaction curves (Figures 6a and b). The decreased diversity was exacerbated by the addition of a FOS-supplemented diet at 14 days (ICR-C: 3.66±0.11 v. ICR-FOS 1.77±0.59 $p=0.02$) (Figure 5.6a). Fourteen days following ICR, the Firmicutes phylum dominated (Figure 5.8; Table 5.1), and the dominant family in both ICR-C and ICR-FOS groups was *Lactobacillaceae* (Figure5.9; Table 5.1). However, *Lactobacillaceae* accounted for nearly all reads in the ICR group supplemented with FOS (90.6 ± 4.9%), while a more modest level was seen in the ICR-C group (52.6 ± 4.7%). The increase in *Lactobacillaceae* was at the expense of *Enterobacteriaceae* and *Enterococcaceae*, which were relatively more abundant in ICR-C mice at 14 days (Table 5.1, Figure 5.10). 28 days following ICR, the relationship between FOS-supplementation and decreased diversity persisted (ICR-C: 2.77±0.37 v. ICR-FOS: 1.82±0.38 $p=0.11$) although interestingly by this point ICR-FOS mice showed an elevation in Bacteroidetes, which was not seen in the

ICR-C mice (Table 5.1). ICR-FOS mice also saw an increase in Proteobacteria, which was not seen in ICR mice. There was a correlation between the loss of diversity and bifidogenic effectiveness of FOS as measured by qPCR ($r= -0.61$, $p=0.047$) (Figure 5.6d).

Fecal Short-chain Fatty Acids

The concentration of short-chain fatty acids (SCFA) was measured in the stool at day 28. As expected, FOS-supplementation led to an increase in the total concentration of fecal SCFA ($p<0.01$), and acetate ($p<0.01$) (Figure 5.7). ICR depleted fecal acetate, propionate, and total SCFA, and completly eliminated fecal butyrate (Figure 5.7). Acetate is an endproduct of FOS metabolism by bifidobacteria, and accordingly there was a correlation in ICR mice between the proportion of fecal bifidobacteria and the level of acetate in the stool ($r=0.76$, $p<0.01$).

Discussion

In this study, we demonstrate that FOS-supplementation of a post-ICR diet results in a bloom of *Lactobacillaceae* and subsequent decrease in fecal bacterial diversity along with an impairment in barrier function and increased inflammation in IL-10-/- mice.

Recurrence of Crohn's disease following ICR has been a challenging problem. Recently there has been a linkage identified between certain bacterial populations and the loss of microbial diversity seen in CD disease recurrence (De Cruz, Kang, *et al.*, 2015; Mondot *et al.*, 2015). A study by Fedorak *et al.* demonstrated the potential of microbiota manipulations, in the form of the probiotic combination VSL#3, in altering the course of post-operative CD recurrence (Fedorak *et al.*, 2015). Our model showing massive gut microbial changes induced by surgery is consistent with previous literature (Devine *et al.*, 2013; Perry *et al.*, 2015). This consisted of a decrease in total bacterial density following ICR, and a stark decrease in both bacterial diversity and richness. This was associated with the loss of specific bacterial groups, including bifidobacteria following ICR. These microbial changes were associated with the loss of beneficial microbial metabolites, especially butyrate, which was not present in the feces of post-ICR mice. These changes were accompanied by an apparent worsening of barrier function. Ultimtely ICR was associated with increased expression of systemic and enteric pro-inflammatory cytokine expression.

In previous studies, FOS-supplementation has been show to stimulate the growth of Bifidobacteria, increase the production of butyrate, and decrease colonic inflammation (Winkler, Butler and Symonds, 2007; Koleva *et al.*, 2012). Bifidobacteria have also been shown to decrease the secretion of inflammatory cytokines by intestinal epithelial cells

and by immune cells (Riedel *et al.*, 2006; Heuvelin *et al.*, 2009; López *et al.*, 2010). Based on these findings, we attempted to improve the delterious course of post-ICR inflammation in a murine model of post-operative Crohn's disease recurrence by inducing the growth of protective microbes through supplementation of the diet with FOS. In terms of bifidogenesis, FOS was successful; the relative amount of bifidobacteria was higher in mice whose diets were supplemented with the prebiotic. It has been suggested that the beneficial effect of bifidobacteria may be mediated by the increased production of SCFA (Fukuda *et al.*, 2011). However, although FOS-supplementation was successful in increasing fecal SCFA concentrations, particularly in non-surgical mice, there was no increase in butyrate production in ICR mice. Indeed, despite a small increase in acetate induced by FOS feeding in the ICR mice, the overall condition of ICR-FOS mice was worsened compared to ICR-C mice, as evidence by elevated colonic, ileal, and systemic inflammatory cytokines and a delay in the restoration of weight following surgery.

The unintened consequence of heightened inflammation following FOS-supplementation post-ICR may be due to the effect of the fibre on overall microbial diversity. FOS-supplementation exacerbated the loss of diversity and richness induced by surgery, and in fact the effectiveness of FOS-supplementation in increasing bifidobacteria post-ICR actually correlated with a decrease in bacterial diversity. *Lactobacillaceae* appeared to benefit most from FOS-supplementation following ICR. Both of the selective pressures applied during this study, namely ICR and FOS-supplemenation, proved to enhance the fitness of *Lactobacillaceae*, and led to this family becoming overwhelming dominant following ICR in FOS-supplemented mice. A loss of microbial diversity in the gut has been associated with many health conditions, including

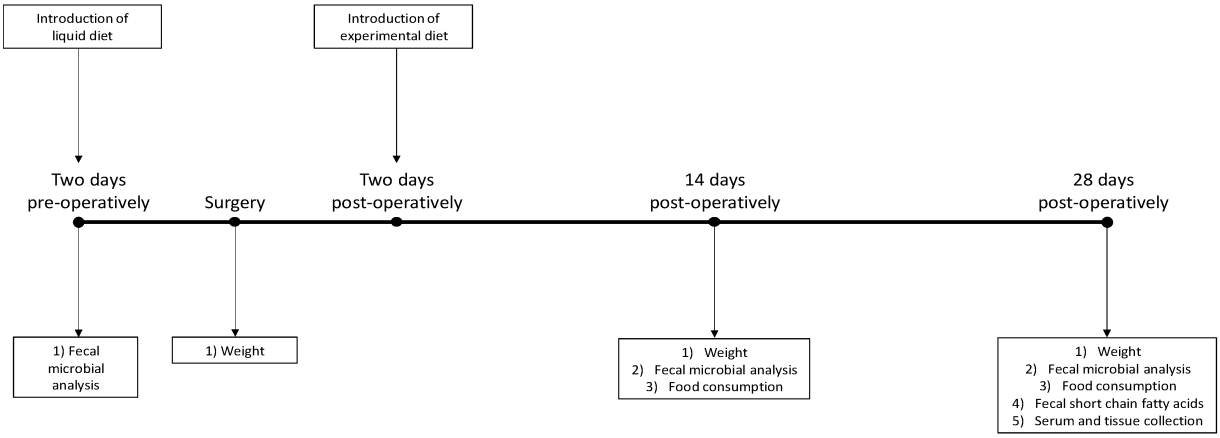
recurrent C. *difficle* infection, psoriatic arthritis, and Crohn's disease (Manichanh *et al.*, 2006; Chang *et al.*, 2008; Scher *et al.*, 2015). Adequate diversity levels are considered necessary for the function of an ecosystem. The importance of diversity is almost certainly multifactorial, though one mechanism may be the production of specific metabolites that are only possible through crossfeeding. Crossfeeding is the process where the breakdown products of polysaccharides from primary degraders are used as secondary substrates by different microbes (Vuyst and Leroy, 2011). A prominent example of this phenomenon is the increase of butyrate production by the *Faecalibacterium* genera following acetate production by Bifidobacteria (Rios-Covian and Gueimonde, 2015). The reliance of crossfeeding on microbial diversity may explain the pattern of fecal butyrate seen in this experiement. Those mice that did not undergo sugery were able to preserve a high-level of diversity, and consequently had a signifigant degree of crossfeeding resulting in butyrate production. The loss of diversity following surgery crippled the ability of the microbiome to engage in metabolic crossfeeding. Therefore, despite FOS stimulating increased fecal acetate, the overall intestinal ecosystem was not conducive to butyrate formation. The route of FOS-administration in this project was through solid diet, as opposed to liquid oral gavage. Liquid FOS may increase the rate of passage through the stomach, and lead to altered downstream effects of FOS, a factor that must be considered in future works (Moreau *et al.*, 2003; Winkler, Butler and Symonds, 2007).

The systemic and local inflammation seen following ICR and FOS-supplementation may have been partially due to impaired barrier function. This effect has been previously demonstrated following FOS-supplementation(35). In this work FOS-supplementation decreased the expression of two tight junction proteins, *occludin* and

ZO1, and led to an increase level of serum LPS suggesting a loss of barrier function. Interestingly, ICR-mice on the FOS supplementation also had an increase in Proteobacteria by day 28, suggesting that FOS induced a more favourable lumenal environment for the growth of these potentially inflammatory organisms.

The immediate post-operative period in CD has often been viewed as a favourable time in which to initiate therapies, as it is seen as a clean slate, devoid of disease pathology. While that may be true in terms of pathologic inflammation, we demonstrate here that in terms of microbial pertubations this is not the case. In reality, the post-operative period represents a time of significant dysbiosis, and treatments initiated in this timeframe must take that into account. In this case, providing a competitive advantage to those bacteria that utilize FOS as a substrate, specifically *Lactobacillaceae*, diminished microbial diveristy and subsequently resulted in worsened barrier function, increased levels of *Citrobacteria*, and increased inflammation. Future attempts at manipulation of bacterial populations in the profoundly dysbiotic gut must address community diversity as well as propagation of specific beneficial species.

Figure 5.1
Experimental design



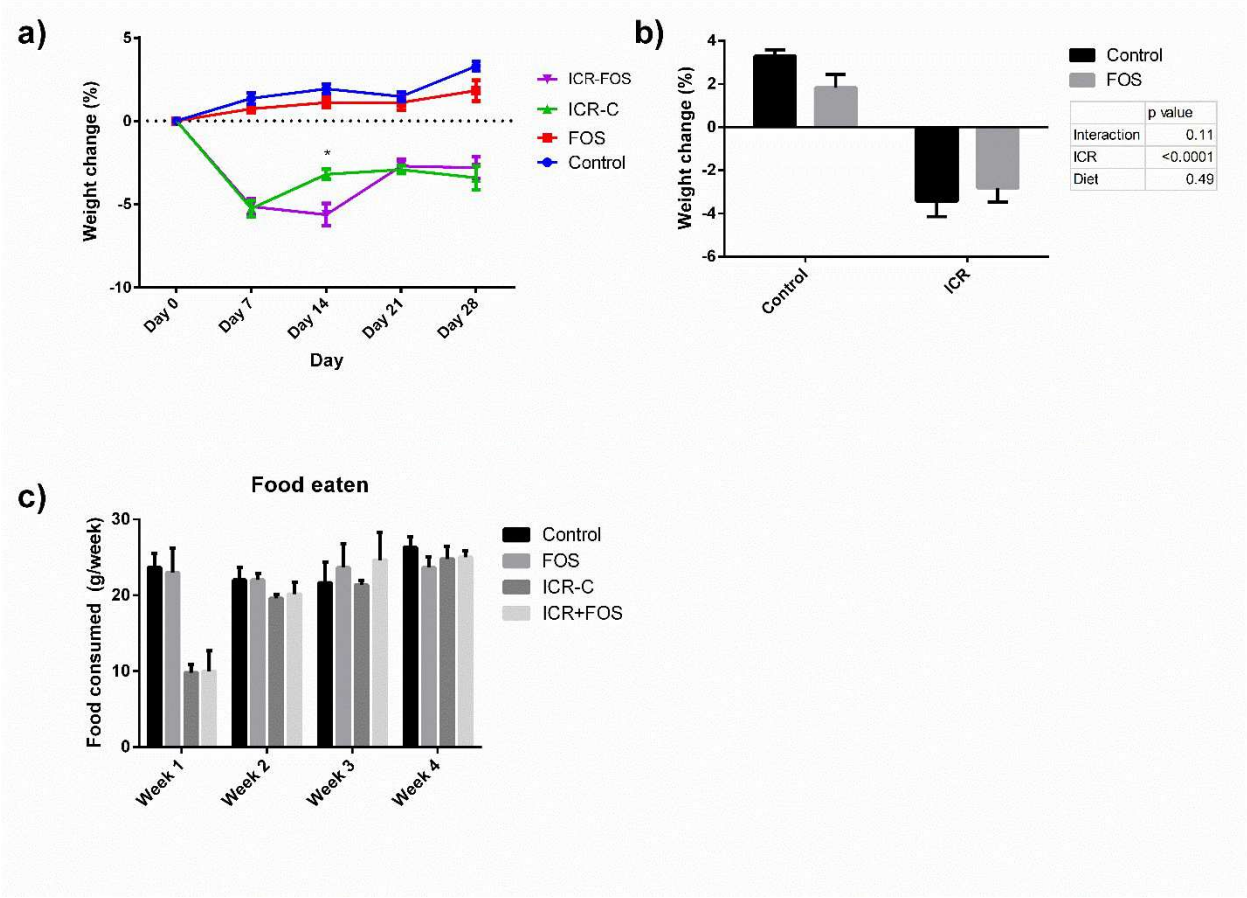


Figure 5.2: FOS-supplementation was associated with delayed recovery following ICR.

a) Mean percent weight change ± SEM from baseline at 0, 7, 14, 21, and 28 days. Mice experienced weight loss following ICR. ICR-C mice experienced recovery more quickly than ICR-FOS mice, with significantly different weights on day 14 (represented by *, Student's t-test, p=0.01).

b) ICR mice lost significantly more weight over the course of the experiment than their non-surgical counterparts (Two-way ANOVA, † represents p<0.0001)

c) Following ICR, mice consumed less food in the first week (Student's t-test compared to control group. † represents p<0.0001). Food consumption was similar in weeks 2 through 4.

Control: n=5; Control-FOS: n=6; ICR-C: n=5; ICR-FOS: n=6

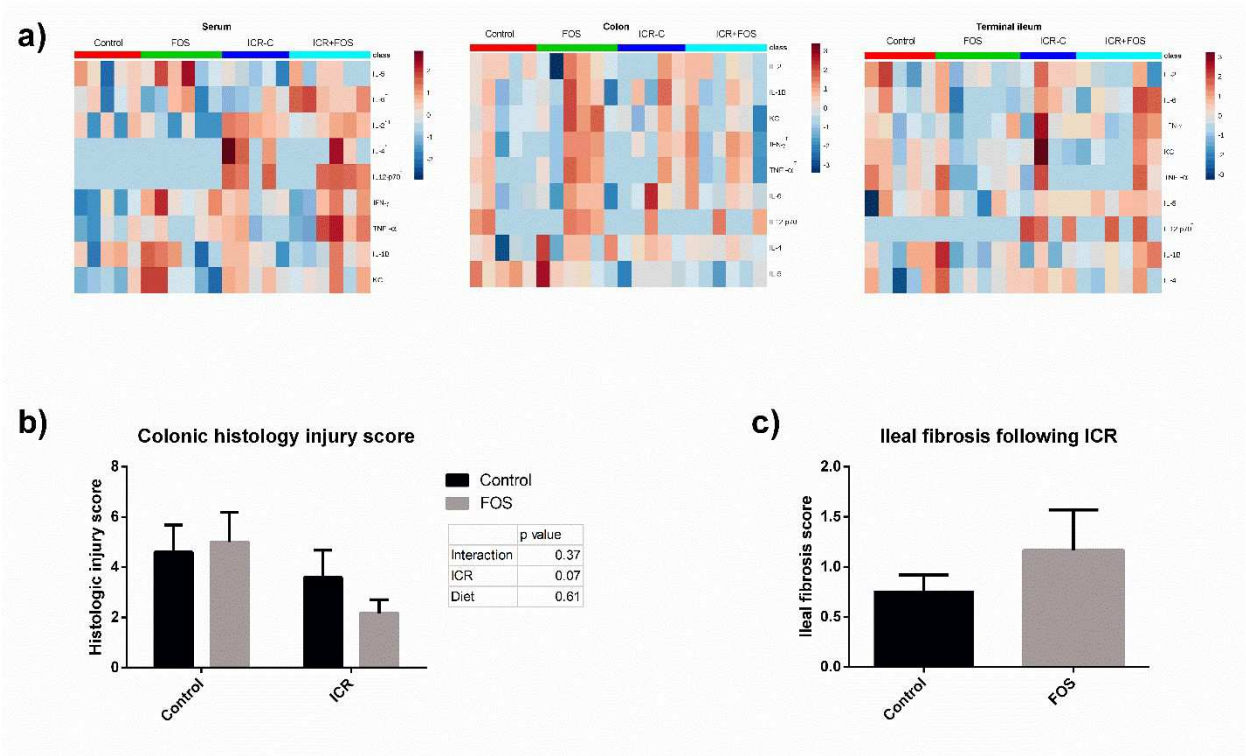


Figure 5.3. ICR and FOS-supplementation led to increased systemic and enteric cytokine expression

a) Heatmap of relative cytokine expression in the serum, terminal ileum, and colon after logarithmic transformation. ICR mice and FOS-supplemented mice expressed significantly higher levels of IL-2, IL-12, and IL-4 ($p<0.05$). (Two-way ANOVA, * represents a change induced by ICR and T represents a change induced by FOS, $p\leq0.05$).

b) Histologic injury scores given as a combined score for enterocyte injury, epithelial hyperplasia, and lymphocyte and neutrophil infiltration into the lamina propria. Two-way ANOVA revealed no effect of FOS or ICR on histologic injury score as a whole or in individual components of the score.

c) Fibrosis score assessing collagen deposition in the TI following ICR. ($p=0.28$)

Control: n=5; Control-FOS: n=6; ICR-C: n=5; ICR-FOS: n=6

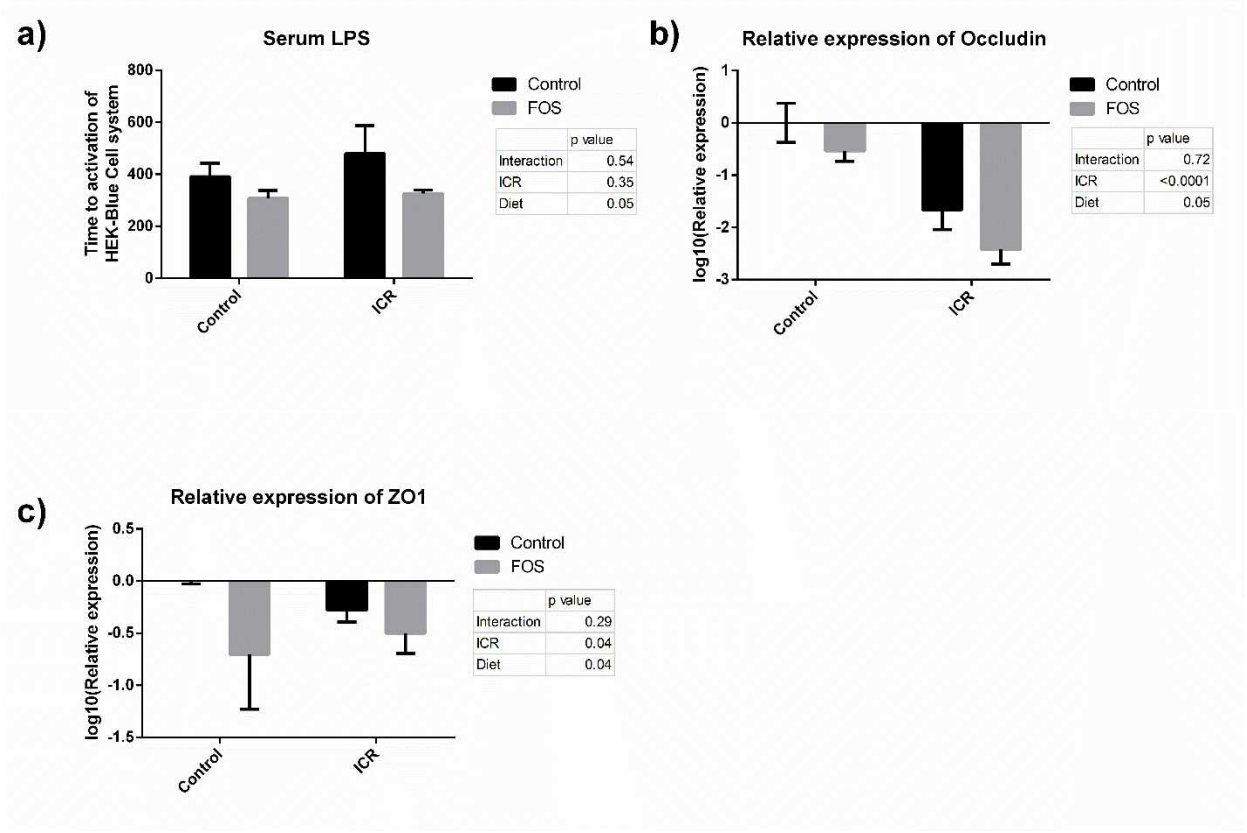


Figure 5.4- FOS-supplementation worsened barrier function

a) Time to activation of a HEK-Blue cell system sensitive to LPS by serum. A lower time to activation of this cell system is associate with higher levels of serum LPS. Presented as mean time to activation ± SEM. FOS-supplementation was significantly associated with increased LPS (p=0.05) (Two-way ANOVA).
b) Relative mRNA expression of *occludin* following logarithmic transformation in the TI of mice presented as mean expression ± SEM. Both FOS-supplementation (p=0.05) and ICR (p<0.01) were associated with decreased expression.
c) Relative mRNA expression of *ZO1* in the TI of mice presented as mean expression ± SEM. Both FOS-supplementation (p=0.04) and ICR (p=0.04) were associated with decreased expression.

Control: n=5; Control-FOS: n=6; ICR-C: n=5; ICR-FOS: n=6

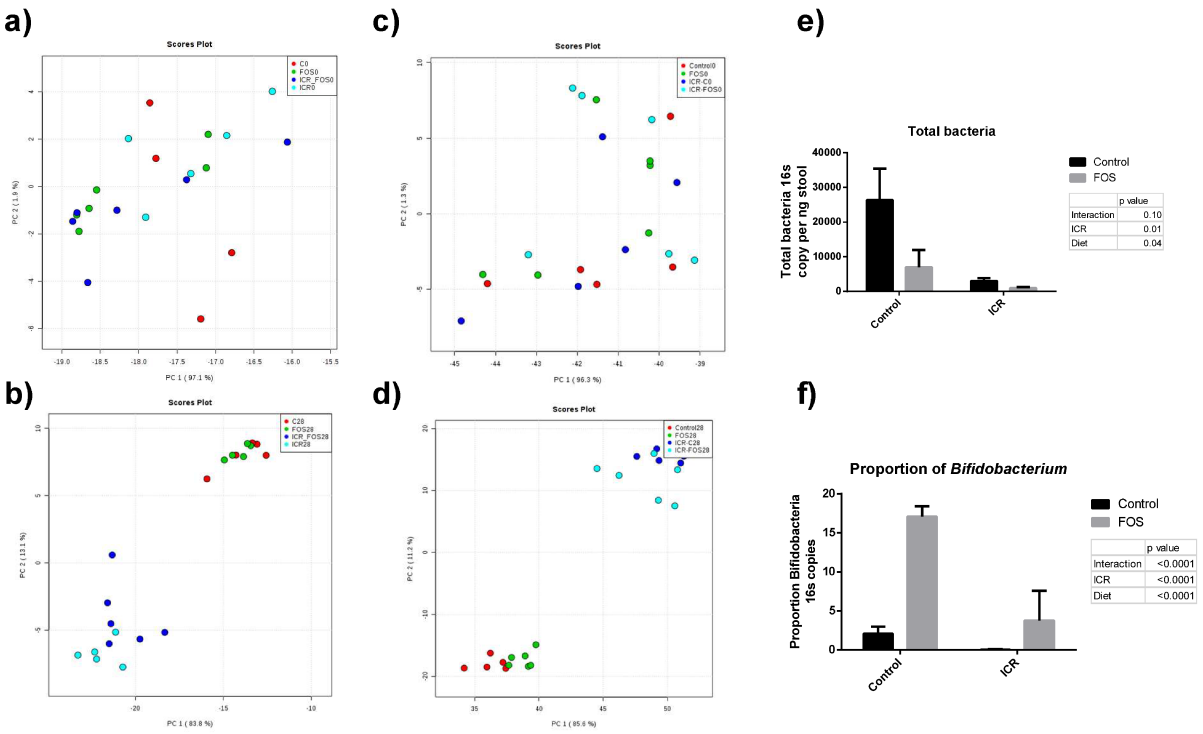


Figure 5.5. ICR causes significant shift in bacterial populations, and a reduction in total fecal bacteria and Bifidobacteria.

a) PCoA plots of the Bray-Curtis index showing clustering at the phyla level at prior to the initiation of liquid diet (a) and day 28 (b) and at the family level at d0 (c) and day 28 (d). (e) Total fecal bacteria per ng of stool in feces at day 28 of all groups. ICR and FOS-supplementation were associated with decreased fecal bacteria ($p<0.01$, $p=0.04$). (f) Relative proportions of bifidobacteria in feces at day 28 of all groups. ICR decreased the proportion of bifidobacteria ($p<0.01$), while FOS supplementation increased the proportion of bifidobacteria ($p<0.01$).

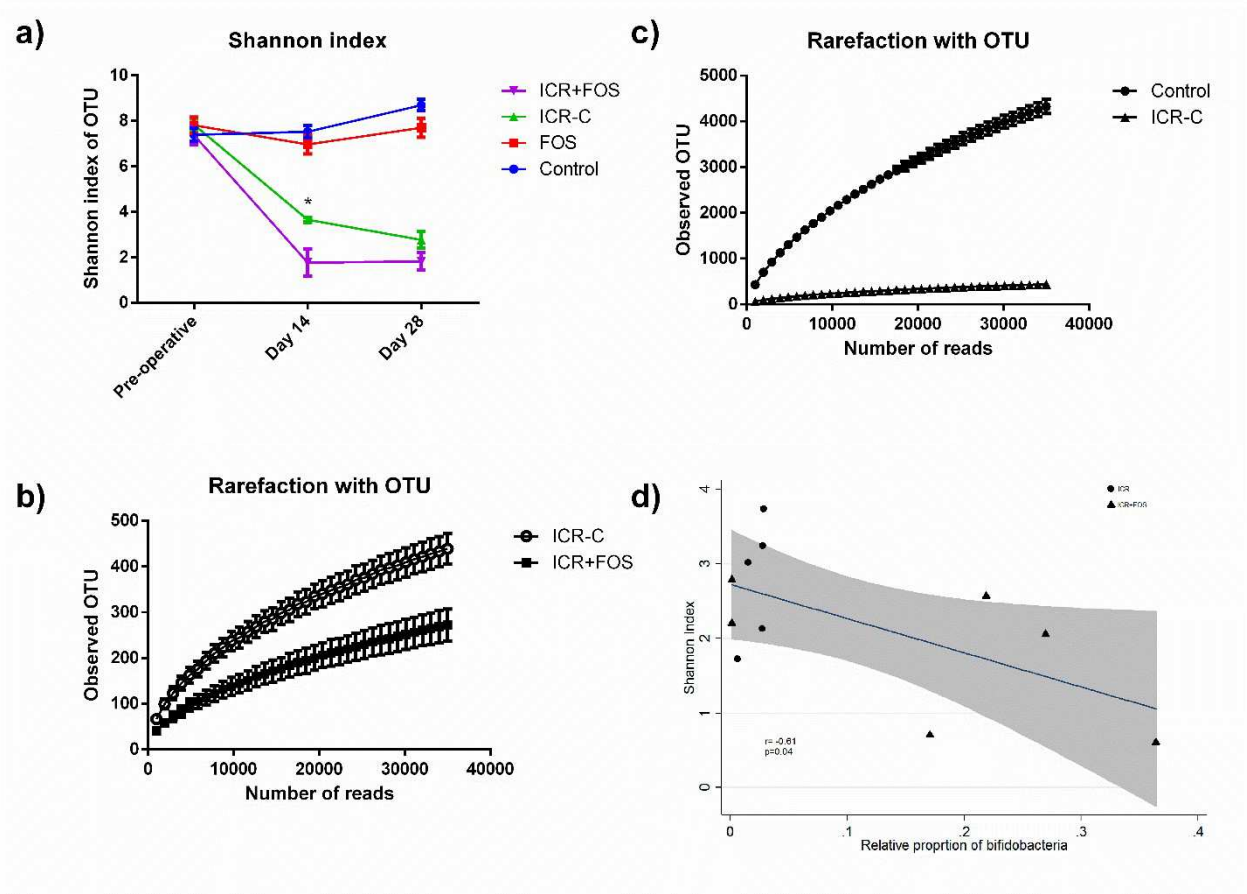


Figure 5.6. ICR leads to a decrease in bacterial diversity and richness that is exacerbated by FOS-supplementation.

a) Mean Shannon diversity index value ± SEM of each treatment group through the course of the experiment. At day 14 ICR-C mice possessed greater diversity than ICR-FOS (represented by *, Student T-test, p=0.02). b) Rarefaction curve representing species richness. Data points represent mean OTU observed at a certain number of reads ± SEM. ICR lessened species richness. c) Rarefaction curve representing species richness. Data points represent mean OTU observed at a certain number of reads ± SEM. FOS-supplementation post-ICR further decreased species richness. d) Plot of Shannon index value compared to the proportion of bifidobacteria in the stool at day 28. As bifidogenesis increased, diversity decreased. (r=-0.61, p=0.05)

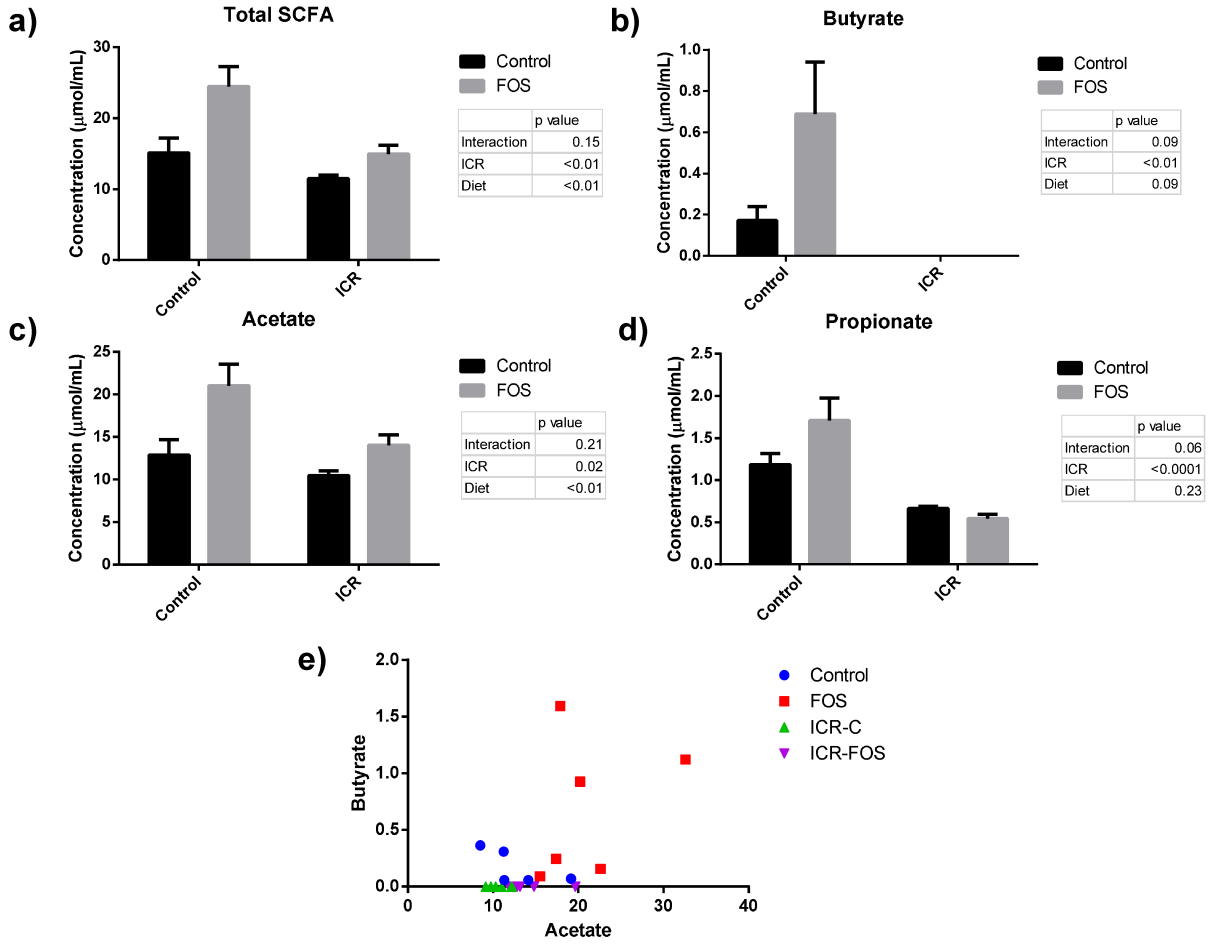


Figure 5.7. Effect of FOS-supplementation on fecal SCFA levels.

(a) Total mean fecal SCFA concentration ± SEM at day 28. ICR was associated with a depletion of SCFA ($p<0.01$), while FOS-supplementation increased total fecal SCFA ($p<0.01$). (b) Mean fecal butyrate concentration ± SEM at day 28. ICR mice had a complete absence of stool butyrate. FOS-supplementation in control mice was associated with a trend toward increased fecal butyrate ($p=0.09$). (c) Mean fecal acetate concentration ± SEM at day 28. ICR was associated with a depletion of acetate ($p=0.02$), while FOS-supplementation was associated with increased fecal acetate ($p<0.01$). (d) Mean fecal propionate concentration ± SEM at day 28. ICR was associated with a depletion of propionate ($p<0.001$). (e) Fecal butyrate was seen following FOS-supplementation in non-ICR mice, but no butyrate was detected in ICR mice, regardless of FOS-supplementation.

Figure 8

Microbial composition pre-operatively and on days 14 and 28 in ICR-C and ICR FOS mice at the Phylum level

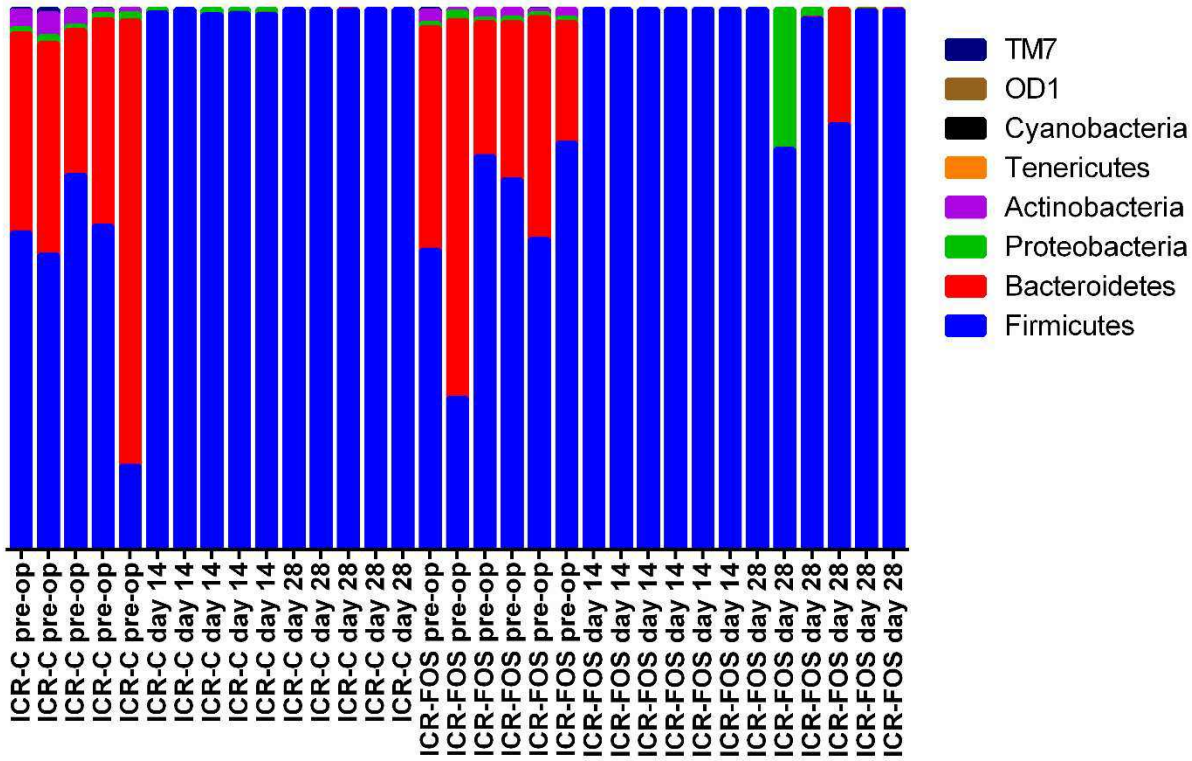


Figure 9
Microbial composition pre-operatively and on days 14 and 28 in ICR-C and ICR FOS mice at the Family level

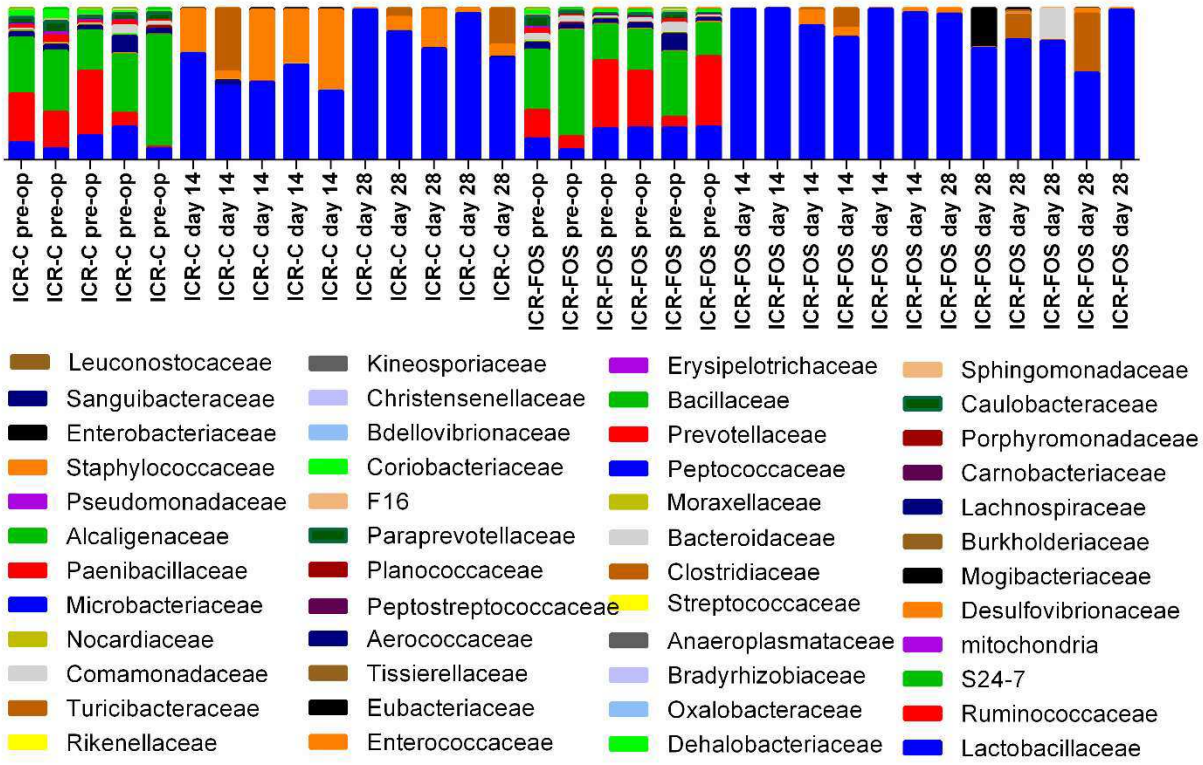


Figure 10
Relative frequency of differently abundant bacterial families in ICR mice over time. * represents a signficant difference following LDA analysis.

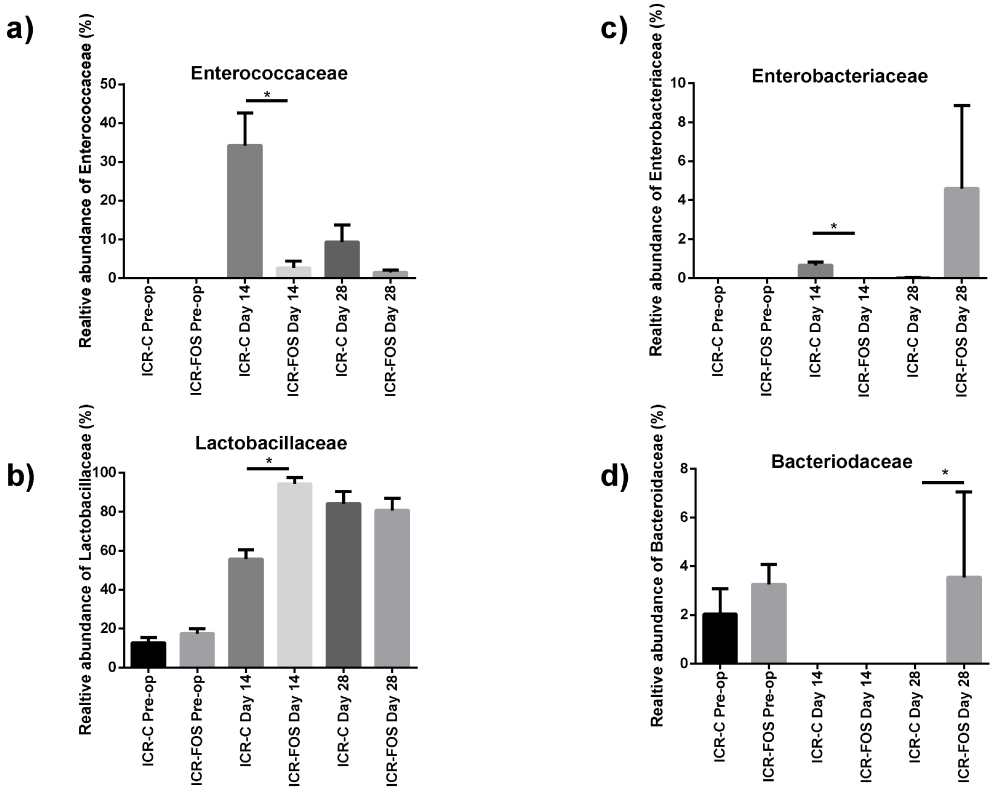


Figure 11
Mean proportions of the five most abundant bacterial phlya present in experimental mice through time

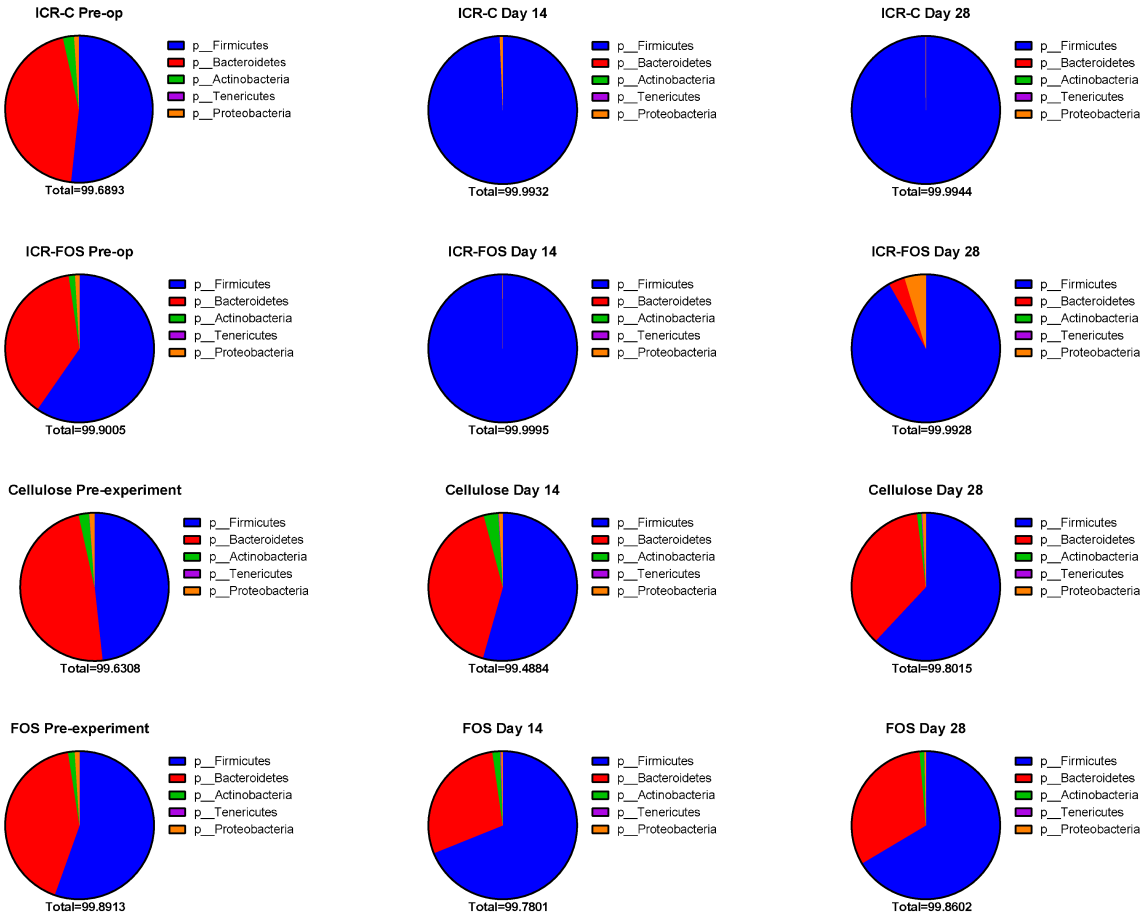


Figure 12
Mean proportions of the ten most abundant bacterial families present in experimental mice through time

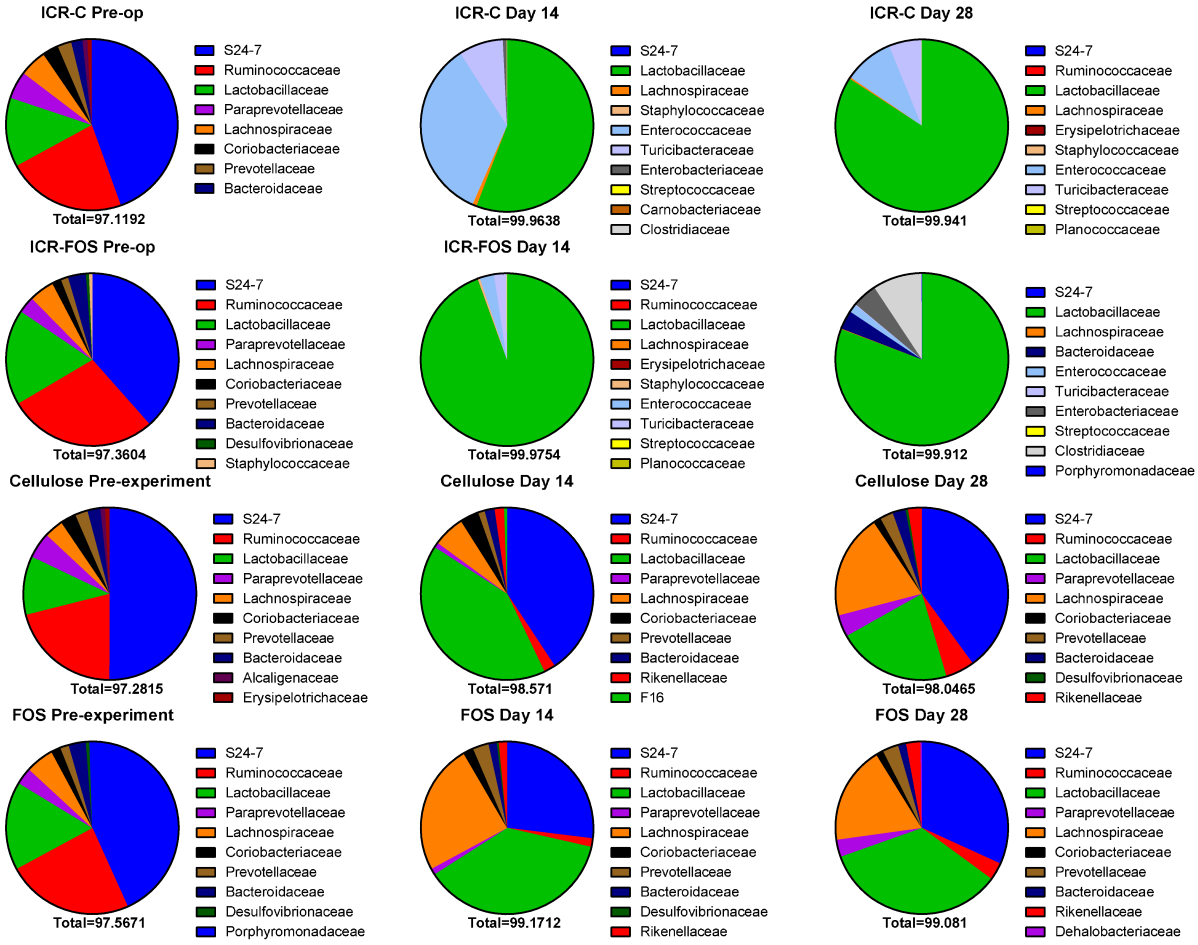


Table 5.1- Effect of FOS-supplementation post-ICR on bacterial populations at 14 and 28 days.

Relative abundance (%)	Day 14 ICR-C (SEM)	Day 14 ICR-FOS (SEM)	LDA effect size	Day 28 ICR-C (SEM)	Day 28 ICR-FOS (SEM)	LDA effect size
Phylum						
Firmicutes	99.10 (0.09)	99.96 (0.01)	3.82	99.8 (<0.1)	89.9 (4.5)	4.81
Bacteroidetes	0.03 (<0.01)	0.02 (0.01)	NS	0.02 (<0.01)	3.51 (3.14)	4.33
Proteobacteria	0.01 (0.0)	0.64 (0.34)	NS	0.03 (0.02)	4.62 (9.51)	NS
Class						
Bacilli	99.35 (0.59)	99.88 (0.03)	3.87	99.54 (0.22)	81.29 (5.91)	NS
Gammaproteobacteria	0.64 (0.15)	<0.01 (<0.01)	3.88	0.02 (0.01)	4.50 (3.81)	NS
Bacteroidia	0.03 (<0.01)	0.02 (0.01)	NS	0.02 (0.02)	3.51 (4.50)	4.54
Clostridia	0.06 (0.05)	0.69 (1.31)	NS	0.28 (0.35)	9.01 (13.95)	NS
Family						
Lactobacillaceae	52.58 (4.71)	90.61 (4.97)	5.27	80.00 (7.21)	78.41 (6.32)	NS
Enterococcaceae	32.8 (7.26)	2.31 (1.37)	5.20	8.77 (3.79)	1.46 (0.51)	NS
Enterobacteriaceae	0.63 (0.15)	<0.01 (<0.01)	3.94	0.02 (0.01)	4.50 (3.81)	NS
Clostridiaceae	0.01 (0.02)	<0.01 (<0.01)	NS	0.01 (0.01)	9.23 (14.5)	NS
Bacteroidaceae	<0.01 (<0.01)	<0.01 (<0.01)	NS	<0.01 (<0.01)	3.45 (3.12)	4.55
Lachnospiraceae	0.77 (1.52)	0.03 (0.05)	NS	0.27 (0.39)	0.08 (0.1)	NS
Genus						
Lactobacillus	52.58 (4.70)	90.61 (4.97)	5.49	80.00 (7.21)	78.41 (6.32)	NS
Clostridium	<0.01 (<0.01)	<0.01 (<0.01)	NS	<0.01 (<0.01)	9.37 (14.7)	NS
Enterococcus	25.79 (6.13)	0.71 (0.41)	5.36	5.05 (2.75)	0.66 (0.31)	4.36
Citrobacter	<0.01 (<0.01)	<0.01 (<0.01)	NS	<0.01 (<0.01)	4.38 (3.72)	4.44
Staphylococcus	0.02 (0.01)	0.42 (0.37)	NS	<0.01 (<0.01)	0.01 (0.01)	4.22

Significant values as calculated using the Linear Discriminant Analysis effect size tool, which employs a Kruskal-Wallis sum-rank test ($p<0.05$), a Wilcoxon rank-sum test ($p<0.05$) and subsequent linear discriminant analysis to identify and estimate the effect size of each differentially abundant population.

NS- not significant

Chapter 6- Conclusions

This project aimed to identify the role of immunologic, genetic and microbial factors in the recurrence of post-operative CD and to determine if disease recurrence can be modulated through dietary intervention. Presented here are four projects conducted to this end. First, the microbiota of a cohort of CD subjects was assessed longitudinally from the time of intestinal resection to ileocolonoscopy. This assessment included microbial profiling at the time of surgery and 6 months post-operatively. Second, we examined genetic loci associated with CD, and the effect of SNPs at these loci on the risk of surgical recurrence. Third, we assessed the inflammatory milieu of the gut at the time of surgery via cytokine concentrations to identify molecules that predict endoscopic recurrence. Finally, a mouse-model of ICR in CD was used to assess for microbial changes following ICR, and to assess for the impact of a prebiotic fibre in the course of microbial recolonization and the inflammation process.

CD is a multifactorial disease. There are genetic, environmental, immunologic and microbial factors that are implicated in its development (Abraham, Cho and Abraham, Clara, Cho, 2009; Baumgart and Sandborn, 2012; McGovern *et al.*, 2015). This makes studying CD a challenge, as there are often multiple phenomena to consider. The special case of post-operative CD recurrence is especially challenging to examine, given the heterogeneity of disease presentations and the impact of varying medical approaches to this timeframe. Nonetheless, addressing post-operative CD recurrence is important, as 80% of the over 4 million CD patients found worldwide will require surgery within 10 years of diagnosis (Olle Bernell, Lapidus and Hellers, 2000; Burisch and Munkholm, 2013).

The relationship between the fecal stream and post-operative CD recurrence was established 25 years ago (Rutgeerts *et al.*, 1991). Since then, a number of specific bacteria have been implicated in disease recurrence, though no causal relationships have been established (Darfeuille-Michaud *et al.*, 2004; Dey *et al.*, 2013; De Cruz, Kang, *et al.*, 2015; Mondot *et al.*, 2015). Other disease processes have been linked to microbial populations, including obesity, liver disease, and non-operative IBD (Turnbaugh *et al.*, 2006; Wright *et al.*, 2015; Boursier *et al.*, 2016). In response to these relationships, therapies have been developed to modulate the microbiota in hopes of altering the course of disease. These include antibiotics, probiotics, and prebiotics. FOS is one such naturally occurring prebiotic found in many common foodstuffs (Rutgeerts *et al.*, 1995; Rivero-Urgell and Santamaria-Orleans, 2001; Lindsay, 2006; Sabater-Molina *et al.*, 2009; Fedorak *et al.*, 2015). FOS-supplementation increases the proportion of certain bacteria associated with decreased inflammation, and SCFA production in the gut (Bakker-Zierikzee *et al.*, 2006; Tan *et al.*, 2014).

Given the aforementioned properties of FOS, we hypothesized that dietary FOS-supplementation following ICR would cause a microbial shift, including an increased proportion of *Bifidobacteria*, and subsequently lessen inflammation. Supplementation was successful in altering the microbiome and increasing *Bifidobacteria*. FOS-supplementation also modulated the inflammatory course post-operatively, but not in a way that was beneficial. Decreased microbial diversity, worsened barrier function, and increased systemic inflammation were all seen secondary to FOS-supplementation. These results reinforce the importance of viewing the microbiome as a whole. Community structure, in this case represented by α-diversity, is critical to maintaining a healthy

microbiota. FOS-supplementation may have altered the competitive ecologic environment of the gut in a way that favored certain saccharolytic strains allowing them to become entrenched at the expense of overall diversity. Future work must take a nuanced view toward microbial manipulation, encouraging both individual populations associated with decreased inflammation, and community diversity as a whole.

This project involved the largest collection of peri-operative CD mucosal-associated microbiota samples to date. Regardless, we were unable to define a common microbial profile at the time ICR, due to the large degree of diversity between individuals (β-diversity). Despite this heterogeneity, we were able to identify important changes between the time of intestinal resection and follow-up ileocolonoscopy. There was an increase in the proportion of the Clostridia class of bacteria, which are able to form endospores. This suggests endospore formation as a potential mechanism of survival in the hostile post-operative gut. Decreases were seen in two families of bacteria sensitive to oxygen, Bifidobacteriaceae and Coriobacteriaceae.

Our examination of specimens taken at the time of surgery in patients who maintained remission compared to those who suffered recurrence revealed a novel relationship between members of the Firmicutes phylum that may play a role in recurrence of post-operative disease. In a murine model we identified two populations of bacteria that can tolerate the oxidative post-operative gut, namely those capable of aerobic respiration, and those capable of spore-formation. We also demonstrated that ICR may induce spore-formation. Finally, we have identified a ratio within the Firmicutes phylum of anaerobic spore-forming bacterial families to families that are capable aerobic respiration that described the rate of recurrence with 76% accuracy. There is a potentially

significant impact of these findings. Should this ratio be found to be predictive in future cohorts, it may enable risk stratification of CD patients at the time of ICR. Furthermore, manipulation of these populations may be beneficial. Increasing the pre-operative proportion of SCFA-producing spore-forming bacteria may allow these species to expand their role in the post-operative biologic niche, and ultimately prevent or decrease the rate of post-operative CD recurrence.

Our investigation into the genetic factors involved in the recurrence of post-operative CD revealed a *BACH2* gene variant (rs1847472) to be associated with surgical disease recurrence. Given the role of *BACH2* in B-cell development and T-cell maturation, this relationship suggests that adaptive immunity may play a special role in the development of recurrence post-operatively. A role for adaptive immunity in post-operative recurrence is further supported by the association of IL-16 (involved in T-regulatory cell recruitment) and IL-5 (involved in B-cell maturation) and disease recurrence (Horikawa and Takatsu, 2006; McFadden *et al.*, 2007).

The microbial influence on the course of post-operative CD is still relatively poorly understood. This work represents a step toward a niche-driven approach to the assessment of the microbiota in CD and ICR. Those microbes that possess the ability to tolerate the post-operative gut have come to the forefront. Namely, members of the Firmicutes phylum that form spores or are capable of aerobic respiration. The exact impact of these bacteria in the recolonization process must be addressed, as does the apparent beneficial effect on diversity at the time of ileocolonoscopy. Finally, to fully understand these phenomena, the immunologic mechanism and genetic background, which allow certain species to take root following ICR must be elucidated.

Future Directions

Technologic and medical innovation have radically altered the way in which we understand and treat disease. The post-operative recurrence of CD is not an exception to this trend, as our understanding of disease pathogenesis has evolved due to the profiling of genotypes, microbial populations, and immune phenotypes that are associated with the disease. Individually these findings provide value, however, a thorough understanding of the disease process requires the integration of genomic, epigenomic, metabolomic, microbial, proteomic and transcriptomic information (Stylianou, 2017). An integrated approach offers the benefit of identifying potentially important pathologic relationships, but to date has been hampered by limited samples sizes due to the difficulty in recruiting appropriate subjects, obtaining the myriad of required biological samples, and the exorbitant cost of processing such samples. Adequate sample size is especially important in these types of investigations, given that these studies are prone to a substantial false discovery rate and that multiple comparison corrections are required to ensure that identified relationships are biologically relevant, and not simply random statistical error. To overcome the issue of limited samples size, collaboration between multiple patient-care and research centres focused on CD will be crucial.

Although understanding the process of post-operative recurrence requires a complex, integrated approach, treatment continues to focus on the modulation of individual factors. For example, immunomodulation in the form of anti-TNF therapies, and microbial manipulation via the use of antibiotics may have a beneficial effect on post-operative recurrence (Rutgeerts *et al.*, 1995, 2005; Carla-Moreau *et al.*, 2015). Future treatments

targeting the microbiome may involve pre-operative microbial supplementation, perhaps in the form of an endospore-rich probiotic, designed to tolerate the surgical insult, and act as keystone species in recolonization of the lumen following resection. Immunologic therapies for post-operative recurrence may target adaptive immunity, and specifically the balance between T-reg and T-effector cells.

Future work regarding endospores and their role in gut recolonization after ICR will require a better understanding of microbial dynamics post-operatively. A timeseries of microbial populations post-operatively, in both murine models and human subjects would shed light on this process. Further, identification of the beneficial anti-inflammatory pathways through which endospore-forming bacteria may exert their effect will be important. Findings regarding the cytokine profiles and genetics of post-operative CD recurrence will require validation from other cohorts, and further exploration in the form of multi-centre collaborations or meta-analysis of previous work. Should BACH2, IL-16, and IL-5 be consistently implicated, B and T-cell function should be assessed in CD patients undergoing ICR, in an attempt to understand their role in post-operative recurrence. Finally, work attempting to influence the microbiota in the post-operative period should exercise caution when employing a single agent. The immediate post-operative period is profoundly dysbiotic, and a mixture of prebiotics and probiotics may be more effective in restoring a healthy community ecosystem.

This body of work has identified individual microbial, genetic, and immunologic factors associated with the recurrence of CD following surgery. Translational work may build upon these findings by targeting the microbial recolonization of the gut following intestinal resection, and maturation of cell lines in the adaptive immune system.

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