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The Human Microbiome: Implications for Health and Disease

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The Human Microbiome: Implications for Health and Disease

by

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Abstract

The human microbiome is the collective genome of native microbial flora that have evolved with us over millennia and which are typically consistent, despite geographic, ethnic and dietary differences around the globe. Studies have revealed that agitation to the normal development of the microbiome during the first year of life, such as with antimicrobial use, can lead to atrophy later on, resulting in the development of autoimmune diseases such as inflammatory bowel disease (IBD). Moreover, dysbioses, such as those seen with *Clostridium difficile* infection (CDI), have an accelerated deleterious effect on patients who have already developed inflammatory bowel disease, potentially resulting in life-threatening complications, including ileus, toxic megacolon, and death.

We composed a review article which presents an outline of the loss of a normal microbiome as an etiology of immune dysregulation and IBD pathogenesis initiation. Moreover, we summarized the knowledge base with respect to the role of a healthy microbiome in terms of its diversity and important functional elements, and synopsized some of the therapeutic interventions and modalities that are being explored as potential applications of microbiome-host interactions.

Additionally, we conducted a systematic review of the literature and a meta-analysis in order to clarify the risk of colectomy among the subset of IBD patients with ulcerative colitis (UC) subsequent to their development of CDI. We determined that UC patients with CDI are indeed at an increased risk of colectomy relative to their counterparts without CDI, but that future better quality prospective studies, ideally with a population-based approach and with long term follow-

up of outcomes, would be helpful to better evaluate this potential relationship under different circumstances.

Preface

Chapter 2 of this thesis has been published as B. Missaghi, H. Barkema, K. Madsen and S. Ghosh, “Perturbation of the Human Microbiome as a Contributor to Inflammatory Bowel Disease”. Pathogens. 2014; 3.

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CHAPTER 1: INTRODUCTION

1.1 The Human Microbiome

The human microbiota consists of the 10-100 trillion symbiotic bacterial cells carried by each individual, primarily in the gastrointestinal tract (GIT) and, more specifically, the microbiome refers to the expression of the genes that these cells harbor (1). As evidenced by the explosion of new literature on this subject over the past decade (2), there has been a progressively high degree of interest in the objective of comprehending the roles that these symbionts play and their impact on human health and disease. Analogous to the fact that the concept of "what it *is* to be human" has thwarted mankind from the beginning of recorded human history, the question "what *is* the human microbiome?" has confounded scientists since the term was first coined by Joshua Lederberg in 2001 (3, 4). Honing in on the exact definition of the human microbiome has been complicated by incertitude with respect to terminology: for instance, the terms "microbiota" (defined as the microbial taxa associated with human beings) and "microbiome" (defined as the collection of these organisms and the expression of their genes) are often used reciprocally (4). Additionally, the term "metagenomics" was originally used to refer to complete DNA sequencing, but now is being applied progressively more frequently to studies of precise marker genes such as the 16S rRNA gene. More fundamentally, however, researchers have been focusing on basic questions paramount to the foundational definition of the human microbiome, including its stability over time both within and between populations in different geographic regions, and whether a person actually has a single or multiple microbiomes.

Historically speaking, the earliest studies focusing on the diversity of the microbiome began with the renowned Dutch scientist Antonie van Leewenhoek, who in the late 1600s, compared his own oral and fecal microbiota. His recorded observations detail the remarkable differences in microbes between these two locales and also between samples from individuals in states of health and disease (5, 6). Thus, one could state that studies delineating the striking differences in microbial flora at different body sites, and between health and disease states, truly date back to the establishment of the field of microbiology itself (4). The major advantage we have today with our modern technologies is not solely the ability to more clearly and easily observe these differences, but to work towards determining *why* these differences exist as well as *how* we may be able to manipulate transformations from one microbiome state to another, as a potential therapeutic modality.

At the time of birth, the GIT of a newborn human infant provides a nascent environment for microbial colonization (7) and, indeed, the initial microbiota that an infant inherits depends heavily on the mode of delivery (8). It has been demonstrated that shortly post-delivery, the bacterial flora of infants delivered vaginally resembles the microbiota of their mothers' reproductive tract, whereas infants delivered via Caesarean section harbor bacterial communities found on the hands of healthcare workers (9). Notably, the acquisition of new elements of the microbiome occurs over the first several months of life and, by one year of age, resembles that of a human adult (10). An interesting case study by Koenig et al (11), following an infant's microbiota over the first 30 months of life, revealed notable changes in gut bacterial flora composition at five time points: starting a breastmilk diet, development of fever at day 92 of life, introduction of rice cereal at day 134 of life, introduction of formula and table foods at day 161 of life, and antibiotic treatment and adult diet at day 371 of life (11). Of great interest, each

dietary change in this study was complemented by corresponding changes in gut microbiota and the enhancement of related genes. For instance, microbiome genes associated with vitamin biosynthesis and polysaccharide digestion became more prevalent as the infant began to receive a full adult diet (11).

The relationship between human microbial flora and the environment is known to be quite dynamic with human bacterial flora flowing freely to and from surfaces that we interact with daily (4). Frier et al. demonstrated that human fingertips can easily transmit signature microbial communities to computer keys and computer mice, and that the structure of these communities can be used to differentiate objects handled by different individuals (12). Principle coordinate plots were able to demonstrate which fingers were typing on which keys, and which people were using which keyboards (12). On balance, this paper demonstrates the truly dynamic nature of the relationship between human microbiota, the environment and different body sites.

Furthermore, evidence is increasing for an undeniable link between a host's microbiota, digestion, and metabolism. Murine studies have shown that dietary changes can lead to significant alterations in microbial metabolism, especially those pertaining to small chain fatty acids and amino acids, in as little time as one week (13). Moreover, in an analysis of humans and 59 other mammalian species, 16S rRNA sequences clustered together carnivores, omnivores, and herbivores in principle coordinate spacing, confirming that diet and phylogeny both influence bacterial diversity and metagenomics (14). Implications have been further explored in revealing that gut microbiota also appear to play an important role in obesity. Germ-free mice that receive a fecal transplant from conventional mice have an increase in fat tissue without increasing food quantity consumption, a finding which has been demonstrated to be due to increased energy extraction from the diet as a result of intestinal bacterial function (15).

Indeed, each passing year over the past one-and-a-half decades has revealed a plethora of new links and associations between human microbiome configurations and possible implications for health and disease. Increasingly powerful technologies and tools have allowed us to collect sequencing data at a revolutionarily low cost, and with the advent of approaches such as fecal microbiota transplantation (FMT), 16S rRNA and whole-genome sequencing, the sky appears to be the limit in terms of our potential to understand the microbiome and to harness its expressions to improve health (4). As we continue to develop an increasingly more detailed and systematic understanding of the factors that underlie the plasticity of the microbiome, our goal of influencing and altering its configuration to treat disease will be realized.

1.2 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the intestinal tract that is associated with abdominal pain, intestinal bleeding, weight loss, and diarrhea. The two most common forms of the condition are Crohn's disease (CD), which can affect any segment of the gut, and ulcerative colitis (UC), which is primarily restricted to the colon and rectum (16). Around 5% of patients have features of both subtypes and are labelled "inflammatory bowel disease unclassified;" additionally, there are several other described atypical forms of IBD, including collagenous colitis, lymphocytic colitis, diversion colitis, and microscopic colitis (17). All of these diseases are associated with considerable morbidity, and place a significant burden on individuals, families and society, accounting for an estimated $2 billion of expense per year in Canada alone (18). The incidence of IBD has increased dramatically in developed countries over the past 50 years and has been linked with modernization and western lifestyles (19).

Environmental risk factors are an essential contributor in the pathogenesis of IBD and are believed to be largely responsible for the rapid increase in worldwide incidence (20). In fact, accumulating evidence has demonstrated that IBD is likely due to an exaggerated or aberrant inflammatory response to intestinal microorganisms in a genetically susceptible host (21). In this light, a growing body of research has highlighted the notable importance of host-microbe interactions in the pathogenesis of this spectrum of diseases (22-24), a fact that we will attempt to further explore and define as a goal of this manuscript.

Both CD and UC most frequently present in late adolescence and early adulthood, with a small second peak in the fifth decade in the case of UC (25). In general, non-bloody diarrhea that lasts for more than 6 weeks makes infective etiologies less likely and should raise suspicion for the potential presence of IBD. Fever, anorexia and nocturnal symptoms can definitely be seen in IBD whereas, of note, such findings are very atypical in the irritable bowel syndrome (IBS) spectrum of disease, which is occasionally a differential diagnosis consideration (17). Roughly 90% of patients with UC report bloody diarrhea, abdominal pain and urgency of defecation, whereas CD typically has a more varied presentation—usually with chronic diarrhea, but also sometimes presenting with abdominal pain and weight loss in 70% and 60% of patients, respectively (26). In some cases, CD patients present with intestinal fistulas, granulomas and/or abscesses, or with bowel obstruction in the context of these types of complications. Notably, in CD the inflammation is frequently transmural in nature, whereas in UC it is typically limited to the mucosa layer. Between 25-50% of patients with IBD, typically those with CD, present with extra-intestinal manifestations, including musculoskeletal findings (arthritis, ankylosing spondylitis) and skin findings (erythema nodosum, pyoderma gangrenosum, and psoriasis) (27). Interestingly, cigarette smoking affects CD and UC in divergent ways: smokers are at increased

risk for CD and tend to have more severe disease, whereas non-smokers (and prior smokers) are at a greater risk for UC.

There is no single diagnostic test for IBD, including either CD or UC, and diagnosis is typically made through a careful history and physical examination in combination with radiologic, endoscopic and histological investigations. Suggestive bloodwork may include: a complete blood count (CBC) that reveals anemia or microcytosis (suggesting iron deficiency or chronic disease) and/or thrombocytosis (revealing evidence of inflammation). An erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) test may also be elevated, as a non-specific marker of inflammation. Celiac disease should be excluded (with antibody testing) as should thyroid disease (with a thyroid stimulating hormone level). In addition, infective diarrhea should be excluded using stool cultures and *Clostridium difficile* testing as well as PCR testing for intestinal parasites, if appropriate. As IBD can lead to malabsorption, it is worthwhile to consider checking serum B_{12}, folate, ferritin levels and transferrin saturation (28). Ultimately, a lower intestinal endoscopy with histological confirmation on biopsy is considered the gold standard modality for confirmation of the diagnosis (28).

Treatment of IBD involves a multifaceted approach, including lifestyle alterations (i.e. smoking cessation for patients with CD), medical management, and surgical interventions. A pivotal advance was the introduction of treatment with biologic medications, including anti-TNF-α monoclonal antibodies, which have been demonstrated to be particularly effective for CD (21). However, this and other related therapies are often limited by a loss of efficacy over time (21). Anti-p40 monoclonal antibodies have been shown, in more recent studies, to be effective in the treatment of both CD and psoriasis (29, 30). The p40 cytokine subunit is common to both interleukin-12 and interleukin-23 and, as such, anti-p40 antibodies can inhibit both pathways.

Though inhibition of interleukin-23 has been reported to be effective in many (but not all) animal models of IBD (31, 32), there are concerns that selective inhibition of this interleukin may lead to dysregulation of T-cell subgroups and possible deleterious consequences (30). More recent basic science research in the field has focused on the use of interleukin-10 producing T-cells and interleukin-10 producing bacteria (33, 34), as well as the use of certain bacterial components and "probiotic" bacteria (35, 36) to reduce inflammation as therapeutic modalities. Indeed, it is clear that the future progress of IBD medical therapeutics will depend heavily on the development of more refined, sophisticated and integrated understandings of the mechanisms of immune homeostasis and the related intestinal host-microbe interplay.

1.3 *Clostridium difficile* Infection

Clostridium difficile is a Gram-positive, spore-forming, toxin-producing anaerobic bacterium that was first described in 1935 as part of the normal flora of healthy newborn children (37). In the 1950s, it was mistakenly believed that the endoscopic finding of pseudomembranous colitis, now virtually pathognomonic for *C. difficile* infection (CDI), was caused by the bacterium *Staphylococcus aureus* or the yeast *Candida albicans* (38). *Clostridium difficile* was later definitively identified as the causal agent of pseudomembranous colitis in humans in 1978 (39).

Since its recognition and, particularly, over the past one-and-a-half decades, there has been a significant increase in the incidence of CDI. *Clostridium difficile* is now widely recognized as the leading etiology of nosocomial diarrhea in the world and is associated with significant morbidity and mortality (40). In fact, outbreaks with rates as high as 22.5 cases of CDI per 100 admissions have been reported (41). Notably, CDI includes a significantly broad spectrum of

illness, ranging from nuisance self-limited diarrhea, which may respond to conservative interventions alone—such as the discontinuation of antimicrobial therapy, to severe and life-threatening disease, resulting in toxic megacolon, intestinal perforation and death (42). Contrary to a common belief held by some physicians, CDI in its most severe form may present with obstipation (as opposed to diarrhea), reflecting ileus or toxic megacolon (38) preceding perforation and life-threatening complications.

Risk factors for the development of symptomatic CDI are multiple, multifaceted and include: alteration of intestinal microbiota through the use of antimicrobial agents (38), use of proton pump inhibitors (PPIs) (43) which reduce the ability of the gastric acid to inactivate spores, poor host immunoglobulin (Ig) levels (mucosal IgA, serum IgG) (44), advanced age (45), underlying condition of the host (46), and exposure to toxin-producing strains of *C. difficile* (46), which generally occurs through the fecal-oral route. An antimicrobial agent's *in vitro* activity against the strain of *C. difficile* to which the patient has been exposed additionally impacts the agent's risk of contributing to CDI; cumulative exposure to antimicrobials and duration of exposure are other important factors (47, 48). The sequence of events resulting in infection, as conceptualized by Gerding and others, requires three "hits" (46, 47, 49). The first hit occurs when patients are made susceptible through exposure to antimicrobial agents due to their effects on bowel microbiota and, thus, the intestinal microbiome. Subsequently, if such patients are exposed to toxigenic strains of *C. difficile* (second hit), disease may or may not develop, depending on the presence or absence of other factors (third hit), the most crucial of which is related to host mucosal immunity (e.g. immunosenescence with increased age or lack of ability to mount adequate colonic IgA and/or serum antitoxin IgG responses) (38, 46).

In 2000, an outbreak of particularly severe CDI occurred in Pittsburgh, PA, U.S.A., and resulted in a two-fold increase of disease rates from baseline, an increased number of colectomies, and increased mortality (45, 50, 51). In Quebec, Canada, a similar increased virulence was noted in CDI cases with a 4.4-fold higher incidence of disease in 2005 than in 1991 (156.3/100,000 vs 35.6/100,000) and a 4.9-fold higher mortality in 2004 than in 1991 (22 vs 4.5%) (51). As in Philadelphia, these increases were accompanied by a concurrent increase in colectomies and prolonged hospital stays (51). Eventually it was determined that a previously uncommon toxin gene variant strain of *C. difficile* was responsible for both of these outbreaks, a strain eventually branded as "BI" by restriction endonuclease analysis (REA), "027" by polymerase chain reaction (PCR), "NAP1" by pulse field gel electrophoresis, and "Type III" by toxinotyping studies (52). The BI/027/NAP1/Type III strain of *C. difficile* harbors particular virulence characteristics that have resulted in its spread and its association with increased mortality.

The main virulence factors of toxigenic strains of *C. difficile* are the toxins themselves (i.e. toxin A and toxin B) (52); toxin A is an enterotoxin and toxin B is a cytotoxin (38). They work synergistically to disrupt the cytoskeleton of intestinal epithelial cells; toxin A loosens the tight junctions between the epithelial cells that line the colon, allowing toxin B, which is roughly 1,000 times more cytotoxic than toxin A, to enter epithelial cells and cause cell death (53). Both toxins are among the largest to be produced by bacteria (270-308 kDa) and are encoded for in a chromosomal region called the pathogenicity locus (PaLoc) (54). In addition to the traditional toxins produced by *C. difficile* strains, BI/NAP1 strains can produce another toxin, called "binary toxin" (42). Binary toxin belongs to a family of binary ADP-ribosylating toxins, consisting of CDTa and CDTb, which modify actin and are believed to both enhance bacterial adherence and translocate CDTa into the cell cytosol (55). Lastly, *C. difficile*, similar to *Bacillus anthracis*,

possesses an uncommon virulence factor which allows it to form spores in response to an unfavourable or hostile environment, enabling the organism to survive for extended periods of time on fomites and within sanctuaries in the GIT. Some genotypically distinct strains of *C. difficile*, including the BI/NAP1 strains, have demonstrated a proclivity for hypersporulation compared with non-outbreak strains (56, 57).

CDI typically presents as watery diarrhea (without blood or mucus) in association with constitutional symptoms, including fever, malaise, anorexia, abdominal pain, cramping and leukocytosis (38). Though hemorrhagic stools may be seen in severe disease, grossly bloody diarrhea generally suggests other etiologies of diarrhea, whether infectious or non-infectious in nature, such as enterohemorrhagic *Escherichia coli*, *Shigella spp*., *Salmonella spp*., *Klebsiella oxytoca*, and IBD. Particularly severe cases of CDI may result in life-threatening complications, including ileus, toxic megacolon and death; as mentioned above, in such cases, diarrhea may not be present. An unexplained leukemoid reaction may be a *sine qua non* of CDI and, therefore, should top the list of potential differential diagnosis considerations in such patients (51). In general, a diagnosis of CDI is based on a clinical presentation consistent with CDI with confirmed microbiological evidence of toxin-producing *C. difficile*, or histopathological findings of pseudomembranous colitis (58).

The initial step in managing a patient with suspected CDI is to stop the offending antibiotic (if at all possible) and to initiate appropriate infection control precautions. Affected patients should ideally be placed on contact precautions in private rooms, though cohorting patients with CDI may be necessary if private rooms are not available. Use of single-use disposable equipment is strongly recommended (if at all possible) and when non-disposable medical equipment is necessary, it should be thoroughly cleaned after use with ≥ 10% concentration hypochlorite

bleach or similar compound (59). Moreover, all clinicians and ancillary health care staff should switch from the use of alcohol-based hand hygiene products to hand-washing with soap and running water, the mechanical action of which is necessary to remove spores from hands. Alcohol does not destroy *C. difficile* spores and, ironically, may lead to hypersporulation of some *C. difficile* strains (60). Careful supportive care of CDI patients is critical and should include fluid and electrolyte replacement; the use of anti-motility agents for CDI treatment is discouraged.

Several drugs have been studied to treat CDI, but vancomycin, the first widely used drug for this purpose, remains one of two medications approved by the US Food and Drug Administration (FDA) for this indication, the other being fidaxomicin (Dificid) which was approved in 2011. Despite changes earlier this year to the Infectious Diseases Society of America (IDSA) CDI treatment guidelines (61), metronidazole remains the initial treatment option for mild-to-moderate disease at our institution with the recommended regimen being 500 mg po t.i.d. for 10 to 14 days. Oral vancomycin 125 mg po q.i.d. for 10 to 14 days may be used as an alternative therapy for non-severe CDI and is preferred for patients who are intolerant/allergic or with contraindication(s) to metronidazole, are pregnant, or for nursing mothers (62). In addition, a failure to respond to metronidazole therapy within 5-7 days requires a change in therapy to vancomycin (63). For severe or complicated CDI, immediate initiation of vancomycin 125 mg po q.i.d. is crucial and some clinicians prefer a dosing regimen of 500 mg po q.i.d. for very severe (fulminant) disease (64). In cases with evidence of ileus or early changes of toxic megacolon, vancomycin may be administered rectally as a retention enema q.i.d. as well, and intravenous metronidazole at a dose of 500 mg IV t.i.d. is often added as an adjunctive therapy. In patients with severe disease, the use of fidaxomicin at a dose of 200 mg po b.i.d. is a sensible

alternative to vancomycin. Fidaxomicin and vancomycin have similar cure rates with an OR 1.17 (95% CI 0.82 – 1.66); however, sustained cure rates between the two drugs reveal an OR 1.75 (95% CI 1.35 – 2.27) in favour of fidaxomicin (65). The greatest limitation to use of fidaxomicin in clinical practice is its considerably high cost. Early surgical consultation is recommended in severe or complicated CDI and is generally endorsed in the context of peritoneal signs, severe ileus and toxic megacolon (66). In more recent years, increasing attention has been progressively shifted to the therapeutic practice of the restoration of a healthy intestinal microbiome in the management of CDI.

Fecal microbiota transplantation (FMT) refers to the infusion of fecal suspension (or the formulation of capsules containing fecal material) from a "healthy" donor with the goal of reconstituting the gut microbiota of the recipient for therapeutic purposes. Multiple studies now demonstrate both the safety and efficacy of FMT in the treatment of CDI, especially those with recurrent or recrudescent disease (67). Moreover, there is a growing body of literature supporting the use of this treatment modality in severe CDI (68). Indeed the FDA has approved FMT as an investigational new drug for use in recurrent or refractory CDI, after failure of standard treatment regimens (69), and this microbiome-mediated approach represents a major breakthrough in our approach to treating this difficult problem.

1.4 Incidence of *Clostridium difficile* Infection in patients with IBD

IBD has not been traditionally considered an important risk factor for CDI (70). However, more recent studies have demonstrated both that IBD is an independent risk factor for CDI, and that CDI is significantly associated with IBD exacerbations (71, 72). In fact, some authorities

currently recommend that all IBD patients who present with an exacerbation be routinely screened for CDI (62). The incidence of CDI in patients with IBD is believed to have increased considerably over the past decade consistent with the general increase of CDI within the general population (71), and a growing body of literature actually supports the possibility that even greater increases of CDI have been seen in IBD as compared to the general population (73). In 2004, 7% of CDI cases diagnosed at one institution occurred in patients with underlying IBD; in 2005, this percentage had increased to 16% ($p < 0.01$) (74). During the same time period, the overall rates of CDI in hospitalized IBD patients at this institution increased from 1.8% to 4.6% (74). Of interest, this increasing incidence of CDI appears to primarily affect patients with UC (as opposed to CD), in one study increasing from 2.4% of admissions in 1998 to 3.9% in 2004; rates for CD over the same time period increased from 0.8% to 1.2% (75). These differences are supported by other studies as well (76), and may suggest a lower incidence of colonic inflammation in CD and, thus, less widespread colonic dysbiosis in this condition (77).

Captivatingly, IBD patients who develop CDI appear to do so without necessarily demonstrating any of the traditional risk factors for CDI. Specifically, they tend to be younger, have less prior antibiotic exposure, and typically acquire the community-associated form of the infection (78). Moreover, they may lack typical colonoscopic features of CDI, and may or may not have typical symptoms of disease (75, 79). Several recent peer-reviewed publications in the literature report that patients with IBD are at an elevated risk for developing CDI upon exposure, and that they may have worse outcomes with CDI, including an increased risk for colectomy and death—particularly with respect to UC patients (78, 80-83). However, the current risk of colectomy, which is the treatment of choice in medically refractory CDI with fulminant colitis and toxic megacolon, is unclear amongst this specific population of at risk IBD patients who develop CDI.

1.5 Objectives of this Research

A quick medical database search on Medline Ovid (or Pubmed) rapidly reveals that the human microbiome, and the intestinal microbiome in particular, is currently a truly exciting and dynamic area of research with a figurative explosion of literature on the subject being published over the past decade. A plethora of basic and translational studies are being conducted to explore the attributes and constitution of a microbiome that is consistent with a state of "health," and those that are found in various states of "disease." A growing knowledge base of what a healthy microbiome looks like and how it can be encouraged and maintained over a lifetime of exposure to different diets, antibiotics and other factors is generally believed to be crucial to human health. Moreover, such a foundation of knowledge leads to a natural desire by both clinicians and scientists to determine ways to manipulate the configuration of these microorganisms (and their byproducts) to either prevent disease or to restore health after an important homeostatic balance has been lost. It is in this spirit that the following projects were pursued, as the primary goals and aims of this manuscript.

The objectives of the research projects conducted were as follows:

1. To compose a detailed review article, defining and outlining the human microbiome, describing the loss of the normal microbiome as an etiology of immune dysregulation and IBD pathogenesis initiation, summarizing the role of a healthy microbiome in terms of its diversity and important functional elements, and concluding with a description of some of the therapeutic interventions and modalities that are now being explored as potential applications of microbiome-host interactions.

2. To conduct a detailed and comprehensive systematic review and meta-analysis estimating the risk of colectomy in UC patients who acquire CDI. It is believed that the completion of such a study both adds an important contribution to the existing literature, given that prior systematic reviews on this topic have been limited in scope and quality, and is also an excellent example of a disease state that is believed to be substantially due to aberrancies in the host-microbe relationship, which may eventually be primarily treated by microbiome restorative techniques.

Statement of Contribution:

With respect to the first objective of this thesis, please note that this review article has been published in the peer-reviewed literature, and that it was first-authored by Bayan Missaghi after completion of a comprehensive literature review. The manuscript co-authors include Drs. Herman Barkema, Karen Madsen, and Subrata Ghosh, all of whom meet the criteria for authorship in that they participated in the conception and detailed editing of this paper. They unanimously agree to the use of the publication in its current form as a chapter in this thesis as well as to its publication in The Vault. Please refer to **Appendix A** to view the accompanying letters of copyright permission.

CHAPTER 2: PERTURBATION OF THE HUMAN MICROBIOME AS A CONTRIBUTOR TO INFLAMMATORY BOWEL DISEASE

2.1 Introduction

The human microbiome is the collective genome of indigenous microbiologic flora that has evolved with mankind over millennia. From the moment of birth, each part of the body becomes colonized by characteristic populations of organisms belonging to each of the three domains of life: Archaea, Bacteria and Eukarya (84, 85). However, the human gut microbiome is particularly distinct in that it is home to a plethora of bacteria, an estimated 100 trillion cells, which is approximately 10-fold more than the total number of human cells (86) and which encodes for 150-fold more unique genes than our own genome (87). The majority of these bacteria cannot easily be cultivated using traditional culture techniques, and it has only been since the application of sequencing technology that the complement of human flora has been described in greater complexity (88). Our species has co-evolved with these micro-organisms in a mutualistic fashion, and we have come to rely on them for many processes including immune system maturation, nutrition, maintenance of intestinal barrier permeability and function, and prevention of infection with pathologic species (86). Indeed, the gut microbiota reside in close proximity to the luminal surface of the large and small bowel, placing them in near proximity to the immune system, and endowing them with a key role in immune function and maturation (89). It has become increasingly clear that this fragile balance of organisms has many important associations with regard to the maintenance of health and the prevention of illness. It was our aim to review some of these concepts with a special emphasis on the role of the intestinal microbiota in inflammatory bowel disease (IBD).

It has been suggested that individuals can be categorized into three distinct enterotypes that are separated by dominance in the proportions of one of three genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3) (90). These clusterings appear to have developed in response to primarily dietary environmental factors (90). In this original study, the authors concluded that the robustness of these enterotypes across populations, as well as across phylogenetic and functional levels, was indicative of the fact that they appear to be the result of a limited number of well-balanced discrete bacterial community compositions (90). However, more recent studies have demonstrated the existence of abundance gradients rather than distinct clustering, and have shown that the type of methodology used to examine microbiome composition can have significant effects on conclusions drawn (91).

2.2 Microbiome Alterations and Inflammatory Bowel Disease Immune Dysregulation

Inflammatory bowel disease is a chronic inflammatory disorder of the intestinal tract that is associated with abdominal pain, intestinal bleeding, weight loss, and diarrhea. The two most common forms of the condition include Crohn's disease (CD) and ulcerative colitis (UC) (92). These diseases are associated with considerable morbidity, and place a significant burden on individuals, families and society, accounting for an estimated $1.8 billion of expense per year in Canada alone (18, 19). The cause of IBD remains unknown; however, it is thought to occur in genetically-predisposed individuals who are exposed to microbial, dietary, and environmental triggers (93). The incidence of IBD has increased dramatically in developed countries over the past 50 years and has been linked with modernization and western lifestyles (19). Environmental

risk factors are an essential component in the pathogenesis of IBD and are believed to be largely responsible for the rapid increase in world-wide incidence (93). **Table 1** demonstrates a diversity of elements, which influence the human microbiome and that may impact the development of IBD.

Table 1. Factors Affecting the Microbiome

Age
Genetics
Diet
Medications (antibiotics, etc)
Smoking status
Obesity
Pregnancy-status

In infants, gut microbial communities undergo radical compositional and functional changes in response to environmental exposures until a relatively stable community becomes established by ~2-3 years of age (94). Establishment of the gut microbiome is influenced by early diet, antibiotic exposure, method of birth, breast milk composition, and genetic background (95-98). Antibiotic use during this period can significantly alter the composition of the gut microbiota and result in long term decreases in diversity and altered trajectories of immune development which can modulate susceptibility to disease in later life (94, 99, 100). Indeed, antibiotic exposure and infections during the first year of life have been identified as independent risk factors for IBD (101-103). This is due to the fact that gut microbiota play an important role in immune regulation and barrier function of the gut, and that consequent alteration thereof may result in the loss of tissue integrity (104). Both the innate and adaptive arms of the immune system have been shown

to play a role in IBD, with altered adaptive immune function being the primary contributor to disease pathogenesis (89). In general, this occurs mainly through increased pro-inflammatory cytokines driven by the T-helper subsets or by lack of effective anti-inflammatory regulatory T-cells (89).

Changes in innate immunity are also important and evidence exists that alterations in innate immune function are the primary driver of dysfunctional adaptive immune responses. It has been demonstrated that intestinal epithelial cells may directly or indirectly recognize luminal micro-organisms, and that toll-like receptors (TLRs) may be essential in the maintenance of epithelial cell homeostasis (105). TLRs are specialized trans-membrane receptors of the interleukin-1 superfamily, which recognize and respond to pathogen-associated molecular patterns (PAMPS). The interactions between such TLRs and their ligands are involved in the clearance of pathogenic bacteria, leukocyte recruitment, epithelial homeostasis and maintenance of barrier immunity. Many TLRs are upregulated in IBD once inflammation is established due to the increased presence of TLR promoter inducers, including inflammatory cytokines and NF-KB (105). A loss of TLR signaling homeostasis in either direction, either an increase or decrease in activity, is thought to play a potential role in IBD pathogenesis (106).

Nucleotide-binding oligomerization domain-containing protein 2 (NOD2), also known as caspase recruitment domain-containing protein 15 (CARD15), is a protein which plays an important role in immune system functioning via recognition of bacterial molecules (such as peptidoglycans) and stimulation of appropriate responses. NOD2 alleles are mutated in approximately 15% of CD patients, and this can result in defective neutrophil recruitment in

these patients as a result of NOD2 and interleukin 8 (IL-8) and NOD2 and TLR receptor pathway interactions (107). This supports the concept that the adaptive immune dysfunction in CD may occur due to a failure to receive an early priming signal to pathogenic micro-organisms, resulting in a failure to clear them and the development of abnormal subsequent immune responses to their antigens. Studies now suggest that CD patients with abnormal NOD2 have an increased susceptibility to the generation of strongly polarized Th1 responses, resulting in an overly aggressive adaptive immune response to common intestinal flora and thereby setting the stage for IBD (108).

The general concept of immune dysregulation has led credence to the "hygiene hypothesis," a theory that has been advanced as a possible explanation for the increased incidence of IBD, which claims that improvements in hygiene—particularly in developed countries—are congruent with a rise in autoimmune illnesses (19, 92). Strachan (1989) was the first scientist to connect this hypothesis to an increase in allergic diseases and believed that a child could be overly protected from exposure to microbes with lack of exposure to these agents, in early childhood, resulting in more widespread clinical expression of atopy (109) due to immune aberrancy. It is for this reason that many scientists now advocate for a moderate approach towards cleanliness in young children (110).

2.3 Microbiome Bacterial Dysbiosis in Inflammatory Bowel Disease Pathogenesis

A growing body of literature implicates the abnormal overgrowth or dominance of particular bacterial species, at the expense of others, in the pathogenesis of IBD. Notably, mouse model studies of IBD have shown protection against the development of IBD in a germ-free environment, and lend credence to the role of gut flora in the pathogenesis of this spectrum of illnesses (111). Furthermore, other studies have revealed that the intestinal flora of patients with IBD have less bacterial species diversity and less stability over time, suggestive of an association between alteration of the human microbiota and development of this type of disease (112). In healthy hosts, *Firmicutes*, felt to contribute in a key fashion to micronutrient metabolism, are amongst the most dominant constituents of the human indigenous gut microflora, accounting for the largest proportion (~75%) of bacteria (85). Several studies to date (113-115) have demonstrated reduced *Firmicutes*, in particular *Faecalibacterium prausnitzii* and *Roseburia,* and increased *Ruminococcus* and *Enterobacteriaceae*, especially adherent invasive *Escherichia coli* (AIEC), in IBD (116-126).

Some research to date has demonstrated an increased prevalence of *E. coli* in the ileum of CD patients (117), and a very recent study has shown the ability of *E. coli* to trigger and potentiate intestinal inflammation in mouse models (127). Despite the association of this particular organism with IBD and, in particular, CD, the precise role of adherent-invasive *E. coli* (AIEC) in the initiation or activation of IBD is not yet fully defined. However, there is evidence for upregulation of microRNAs, which reduce expression of proteins necessary for the autophagy response in intestinal cells (128). This suggests that such infected epithelial cells are prevented from self-degradement and recycling of cell components. One theory is that AIEC strains, having the capability of adhering to and invading both intestinal epithelial cells and macrophages, can

translocate across the bowel wall and continuously activate further macrophages, leading to the formulation of granulomas (117). It is known that micro-organisms that are able to penetrate the bowel epithelial barrier and to bypass macrophage killing can trigger a powerful and long lasting inflammatory response (129, 130). Please refer to **Table 2**.

Table 2. Microbiome Alterations in Inflammatory Bowel Disease

Microorganisms	**Effects**	**References**
Bacteroides spp., *Clostridium* spp.,	Increased numbers associated with ulcerative colitis	Okayasu et al, 1990; Hakansson et al, 2014
Enterobacteriaceae and adherent-invasive *E. coli*	Increased numbers associated with inflammation and cancer	Yang et al, 2013
Bacteroides and *Porphyromonas* genera	Increased numbers associated with inflammation and colon polyposis	Dennis et al, 2013
Enterobacteriaceae and *Bacteroides* spp.	Increased numbers associated with inflammation	Ettreiki et al, 2012

Other research has reported an interesting increased prevalence of other organisms, including *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in the intestinal tissue of CD patients (131, 132). MAP has been defined as the etiology of Johne's disease in cattle, a chronic granulomatous illness that is clinically and pathologically similar to CD in humans (133). Moreover, several studies have revealed that a high proportion of CD patients are infected with MAP compared to persons not having IBD (134). In fact, a fairly recent imputation-based association analysis was quite conclusive in the overlap and correlation shown between susceptibility loci for CD and infection with MAP (135). Unfortunately, however, no research to date has either confirmed or denied the precise role of any particular organisms in IBD. In fact, the source of MAP in humans is felt by some researchers to be either an environmental or zoonotic epiphenomenon. A two-year study by Selby et al. revealed that empiric therapy

typically effective against MAP, with clarithromycin, rifabutin and clofazimine for up to two years, did not provide sustained benefits (136). On the other hand, using the data from this study, one can also refute the conclusions drawn, noting that the antibiotic arm did significantly better than the control arm for as long as antibiotics were administered (137). This is suggestive that MAP may be a significant player in at least a subset of IBD patients, and that this type of therapy may be effective for them. Further study will be necessary to sort out this controversy.

An emerging theory is that a dysbiosis or imbalance of intestinal flora may be a trigger for IBD in those who may be susceptible, a problem which is normally avoided through the maintenance of a healthy microbiome. There are many reasons why the presence of normal healthy commensal organisms prevents this process from occurring. One is that commensal microbes resist colonization by the enteric pathogens listed above, both directly and indirectly (138). There are several ways in which this is achieved. Firstly, the most basic method in which normal microbiota favour resistance against opportunistic infection is via niche competition. This involves competition for sites of colonization and nutrient uptake at host epithelial surfaces (139), and modulation of protective immune responses (138). Another modality by which commensal organisms help resist colonization by bacteria associated with IBD is by promotion of barrier immunity. An example of this would be induction of epithelial production of mucin, secretion of mucosal immunoglobulins and the expression of antimicrobial peptides via host TLR recognition of PAMPs (138) as alluded to previously. Yet a third modality of colonization resistance relates to the anaerobic environment of the bowels, a factor which allows particular gut commensals to harness energy via fermentation of nutrients passing through the lumen, resulting in the production of short chain fatty acids (SCFAs). SCFAs include acetate, butyrate

and propionate, and are resistant starches that are generally not well digested in the upper GI tract (140). SCFAs strengthen the intestinal epithelium by increasing tight junction protein (TJP) production and by increasing transepithelial electrical resistance (TEER) (85) as well as by effecting the immune system directly (141). Thus, it is clear that diet, too, by selecting for particular micro-organisms and by resulting in specific metabolites, has a profound impact on the selection of existing normal intestinal flora.

2.4 The Microbiome, Obesity and Crohn's Disease

The worldwide obesity epidemic has more than doubled since 1980, and this condition is associated not solely with metabolic disorders, including type 2 diabetes and cardiovascular disease, but also with a wide variety of disorders such as cancer, sleep apnea, osteoarthritis and gallbladder disease (142). Though the present view is that the major etiology of obesity is unbalanced energy intake and expenditure in association with genetic predisposition, environmental factors may also play a role. Amongst the important outside factors impacting the host's response to diet is the gut microbiome (143). The initial demonstration of particular alterations in composition between the intestinal flora of obese and lean phenotypes was made in leptin-deficient mice, in which the guts of obese mice contained fewer *Bacteroidetes* and more *Firmicutes* than other mice (144). Subsequent studies revealed that the transfer of obese mouse gut flora to germ-free mice could reproduce the donor obese phenotype in the recipient mice (145) and several recent studies, using pyrosequencing and advanced PCR techniques, have demonstrated that high-fat diets increase the *Firmicutes/Bacteroidetes* ratio and decrease *Bifidobacterium* spp. (146). Moreover, a higher abundance of *Ruminococcaceae* and

Rikenellaceae was seen in leptin-resistant obese and diabetic mice than their slim counterparts (147). Of some interest, it has also been shown that bacterial ATPase complexes correlate strongly with human BMI, further supporting the association between the intestinal flora's capacity for energy harvest and obesity in the host (148). Moreover, adipocytes have been demonstrated to release a variety of proinflammatory cytokines and peptides, and it is believed that visceral adiposity may play an important role in the commencement and maintenance of inflammation in CD (149). Based on a time-trend analysis from data collected between 1991 to 2008, a clear correlation has been shown between increasing weight over time and CD (149).

2.5 Human Gut Microbiome Diversity and Functional Changes

It has recently been discovered that the evolutionary composition of gut bacterial flora, regardless of geography, evolves towards an 'adult' phenotype by the third year of life (150). Furthermore, regardless of geography, interpersonal variation in gut flora is greater between children than between adults (150). Moreover, microorganism diversity increases with age, and there are significant phylogenetic differences amongst populations living in different countries and regions (150). Many of these differences are due to diet and the availability of particular nutrients in the environment with increases in proportions of various bacterial species found to be primarily related to functional requirements. For example, individuals living in rural Africa and South America have gut microbiomes that are more similar to those of herbivorous animals, whereas Western microbiomes are more reflective of those of carnivores (151). Thus, these are clear adaptations determined in response to varying diets in these regions.

Over the past several years some interesting trends were discovered in regards to functions of gut microbiota. Though some studies have shown clear variability in the composition of human microbiota over time, the functional potential of the microbiome remains strikingly stable (152). Using metagenomic approaches, it has been shown that 12% of gut-related metabolic pathways are different between IBD patients and healthy controls versus only 2% of genus-level clades in this context (153). In keeping with taxonomic profiling studies revealing lessening numbers of *Firmicutes*, one metagenomic trend that has been shown is that there is a reduction in butyrate and propionate metabolism genes in CD (153), as well as lower levels of other SCFAs (124). There is also an increase in functions associated with auxotrophic bacteria, including a decrease in amino acid biosynthesis, and an increase in amino acid transfer (153). Given the presence of tissue destruction in inflammatory conditions, these bacteria tend to thrive in such conditions where nutrients are easily accessible in the environment (153). Furthermore, studies have shown an increase in sulphate-reducing bacteria in IBD, an interesting fact when one considers that a mechanism of a common medical treatment for IBD, mesalamine, is the inhibition of fecal sulphide production (154). Genes that take part in the metabolism of the sulfa-containing amino acid cysteine are increased in IBD, as is increased sulphate transport (153).

2.6 Impact of Cigarette Smoking on the Microbiome

Other environment factors also impact the gut microbiota. Smoking is an important risk factor in IBD pathogenesis with most studies showing a protective effect of cigarette smoking in UC and a deleterious effect in CD (155, 156). Smoking cessation induces prominent alterations in the composition of normal intestinal flora (157). After cigarette cessation, comparable shifts have

been seen in flora composition as have been described in obese versus lean humans and mice (144, 158); specifically, an increase in the proportion of *Firmicutes* relative to *Bacteroidetes*. In one study of the human microbiome during smoking cessation, a mean weight gain of 2.2 kg occurred over the observation period in the absence of alterations in total calorie intake or other dietary changes (157), suggesting a role of commensal organism changes and consequent metabolic alterations in pathogenic weight gain post smoking cessation. However, the precise molecular and cellular effects of smoking and smoking cessation on gut microbiota continue to be relatively poorly understood and are an area for further detailed study.

2.7 Impact of Antimicrobial Therapy on the Microbiome

It is well known that, since the 1950s, antibiotics have been liberally used in the agricultural industry as growth promoters, though the mechanism for this effect has been poorly understood. Notably, in Canada and the U.S., the heaviest use of such antimicrobials is within farms, with restricted doses fed to livestock in order to increase their growth by up to 15% (159, 160). Interestingly, these effects have shown to be relatively consistent across a broad variety of vertebrate species, including mammals (cattle, swine, sheep) and birds (chickens, turkeys), and follow oral administration of these antibiotics, suggesting that the gastrointestinal microbiota may be a notable target (161). Moreover, the fact that these effects are not agent specific and that this has been shown true for macrolides, tetracyclines, penicillins and other agents, indicates that these growth effects are not side effects associated with a particular class of antimicrobials. Within this context, it has been postulated that sub-therapeutic administration (STA) of such molecules may modify the structure of the gut microbiome and its metabolic function to a significant extent (161). In fact, we know well that antibiotics can have a great effect on the

composition of the microbiome in comparison to other environmental factors. As alluded to earlier, antibiotics administered within the first year of life are believed to cause atrophy of the microbiome and to increase the risk of future autoimmune disease as a consequence (101).

Interestingly, murine model studies have confirmed that STA of antimicrobials can both increase adiposity in mice as well as increase hormone levels that play a role in metabolism by altering the proportions of various microbiota. Specifically, one recent novel study showed that an increase in relative concentrations of *Firmicutes* compared to *Bacteroidetes* in mice treated with STA of antibiotics (161). Perhaps even more interestingly, STA of these molecules were shown in this study to stimulate adipogenesis by increasing SCFA levels and, thereby, by providing direct energy to colonocytes (161), confirming some of the functional pathway alterations that occur as a result of antimicrobial administration. This loss of equilibrium and homeostasis may become substantial when prolonged, and may result in changes in host health and subsequent disease (162). Indeed, murine studies are currently being explored to define models for a variety of diseases related to microbiome shifts and alterations as a result of antimicrobial use.

2.8 Therapeutic Approaches and Applications of the Microbiome

Though antimicrobials have a well-established role in the treatment of septic complications of IBD, their benefit in treating the underlying disease process is more controversial. A number of controlled trials and observational studies have been published over the last 30 years regarding the use of antibiotics in treating CD (163-165). Most of these are small studies of short duration and with significant methodological disadvantages, including the use of different endpoints and

outcomes. However, a 2011 meta-analysis of randomized controlled trials (RCTs) revealed superiority of antibiotics to placebo in the induction of remission of active CD as well as in the maintenance of remission (166). Another meta-analysis of 10 randomized trials in 2012 demonstrated an improvement in symptoms in IBD patients treated with antibiotics as opposed to placebo (167). Regarding UC, the 2011 meta-analysis showed a modest benefit in induction of remission of active disease via antibiotics relative to placebo (166), though less promising than in CD. In reference to the 2007 study by Selby et al (136), though anti-tuberculosis treatment has shown some benefit in CD, it is unclear whether this is as a result of the treatment of MAP, the treatment of other commensal organisms or via other nonspecific anti-inflammatory effects. In terms of the choice of antimicrobial regimen, though there is significant variation and little standardization in the literature, most would advocate for the use of a combination of ciprofloxacin and metronidazole based primarily on clinical experience.

Probiotics are "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" (168). Indeed, several studies in mouse models have demonstrated prevention of the onset of colitis and reduction of inflammation in established disease using *Lactobacillus* spp. and mixed culture supplementation (169-171). However, clinical data on the use of probiotics in humans is limited and most studies have not been placebo controlled (172). Moreover, more study is needed on the properties of different bacterial strains for various indications, as well as information on combinations, dosage and duration of therapy. A related intervention involves the use of prebiotics, food ingredients that are not digested or absorbed in the upper GI tract and which are fermented in the lower GI tract, promoting the growth of micro-organisms with beneficial effects. For instance, inulin and oligofructose are fermented in the

large intestine and encourage the growth of native *lactobacilli* and *bifidobacteria* among other organisms, effects which are associated with reduced mucosal inflammation in animal models of IBD (173).

Fecal microbiota transplantation (FMT) is a procedure which has rapidly grown in acceptability and popularity over the past decade. In the animal kingdom, coprophagy (consumption of feces) is quite common and observed in a variety of species (174, 175). The practice of transfaunation, the transfer of gastrointestinal contents between animals, has been practiced for centuries by veterinarians and, whether conducted naturally or artificially, contributes to accelerated maturation of the gut, enhanced digestion of nutrients and increased resistance to colonization of the gut by pathogenic organisms (176). Historically, despite an innate repugnance for stool, human civilization has used variations of FMT for thousands of years. The earliest known documentation of this comes from Chinese medicine, referring to the use of various fecal preparations for the treatment of human gastrointestinal illnesses (177). However, there are also examples of such practice in other parts of the world including Europe in the late 1600s, where Dr. Franz Paullini, a German physician, published a book on the uses of human and animal feces entitled *Hailsame Dreck-Apotheke* (Salutary Filth-Pharmacy) (176).

In modern times, FMT has been hailed as an extremely effective therapeutic procedure for treatment refractory *Clostridium difficile* infection (CDI) (178). At this point in time, the FDA is allowing the use of FMT for CDI that remains refractory to other therapeutic modalities (69). Given the success of FMT for this indication, it would be sensible to believe that this procedure or derivatives thereof may eventually be applied to several other medical conditions on a more

regular basis, including IBD. Some researchers now advocate for the streamlining of FMT by such interventions as “auto-banking,” in which a sample of patients’ stool is collected and frozen on admission to an inpatient facility for later implantation after completion of antimicrobial therapy (179). Though theoretically reasonable, such methods will certainly require further study and approval by regulatory bodies.

In the context of increasing evidence of the importance of our normal microbiota for the maintenance of health and prevention of disease, there has been a recent explosion of interest in modulating the microbiome to restore balance and promote health. One such application, a derivative of FMT, has been labelled as ‘Microbial Ecosystem Therapeutics’ (MET) and involves the replacement of a damaged and/or dysfunctional microbiota with a fully developed and “healthy” constellation of intestinal bacteria in a synthetic fashion (180). As opposed to traditional probiotic therapies, which involve the use of a single strain or a few strains of bacteria, MET involves the application of entire intestinal bacterial populations, which more closely resemble our own normal flora. The potential implications for such therapy are vast and include the treatment of CDI in addition to IBD, irritable bowel syndrome (IBS), obesity and metabolic syndrome, and even certain types of autism (180).

However, it should be noted that at present the use of FMT or MET for non-CDI indications is an area of intense study and that the optimal approaches appear unclear. Despite anecdotal reports of successful treatment of UC and CD (181, 182), others, including Kump et al, have reported no clinical improvement in small series of patients with UC (183). It is clear that this is a ripe area for further research.

Moreover, SCFAs, the main metabolic products of anaerobic fermentation in the bowel, have been recognized as important mediators in intestinal epithelial barrier permeability and immune function (141). Indeed, higher concentrations of such fatty acids in the intestines and in the blood have a direct impact on reduction of predisposition to or intensity of inflammatory conditions, including IBD and cancer (141). In this context, many researchers are now considering the potential therapeutic applications of SCFAs and their derivatives in the treatment of IBD (184-186). Though results have been quite positive overall, further study is necessary to establish the dose, frequency and form of SCFA products to receive ideal results. Yet another implication of this research is the use of potential SCFA receptor agonists to achieve desired effects in terms of strengthening intestinal barrier permeability and priming the immune system.

Though the use of antimicrobials leading to selection of multi-drug resistant organisms (MDROs) is a growing cause for concern, many researchers would now argue that the likelihood of such medications to disturb indigenous flora, facilitating the spread of prior selected resistant strains or their resistant traits, is even more important (179). This pressure on the microbiome will, of course, not only impact individual patients but the whole of society as MDROs continue to spread as a result. Several important studies have now demonstrated that our current antibiotics, though effective as anti-infective agents, can cause significant collateral harm to our microbiome (187, 188). In this context, another important application of the microbiome in therapeutics involves studying the impact that each of our current antimicrobial classes makes in this realm, and to put stewardship measures in place to favour the use of drugs that have a low microbiome damage profile. This approach could be further expanded to the preclinical drug

approval process to ensure that our drugs in development have the lowest possible impact on the status of our normal commensals (188). Not only will this aid in improving the outcomes of individual patients, but will also help with infection control efforts to prevent and/or shorten the duration of MDRO colonization.

Furthermore, other researchers have advocated for the use of specific medications to be given after an antimicrobial course is complete in order to break down or bind any residual drug that will only serve to further damage the microbiome (189, 190). Indeed, there is a time lag of several weeks to months (and sometimes longer) between the final administration of antimicrobials and reconstitution of the microbiome to pre-treatment levels. This delay, in animal models, is linked with an increased propensity to secondary infections (191).

As we look to the future and as more knowledge is gained regarding host-microbiome interactions and the biochemical and immunologic interactions that lead to the release of antimicrobial peptides and maintenance of barrier immunity, desired effects may be achieved through novel modalities and approaches. Perhaps, we may one day manipulate the host into producing the peptides produced by an intact and healthy microbiome through the application of bacterial antigens or, better yet, the administration of purified peptides and other protective molecules without the need for application of live micro-organisms (179). This will allow healthcare providers to artificially assist with healing the host intestinal barrier without subjecting the patient to the fecal transplant-associated risk of pathogen transmission and will avoid the inherent aversion to receiving a FMT.

2.9 Conclusions

Environmental factors, including the microbial composition of the gut, may be as important as genetic determinants in the pathogenesis of IBD such as CD and UC. However, many details are yet to be determined. Despite this, it is clear that IBD disease pathogenesis involves a combination of an aberrant immune system along with dysbiosis in the gut microbiome. Further study and a better understanding of important host-microbial interactions will allow us to accelerate our development of novel therapeutic applications such as FMT and MET, the use of innovative scoring systems for the impact of antimicrobials on the microbiome, and the hopeful eventual use of purified peptides and other molecules. The ultimate goal will be to optimally restore the normal and healthy mutualistic balance between the host and microbiota, which has existed for millennia.

CHAPTER 3: RISK OF COLECTOMY IN ULCERATIVE COLITIS PATIENTS WITH CLOSTRIDIUM DIFFICILE INFECTION: A SYSTEMATIC REVIEW AND META-ANALYSIS

3.1 Introduction

IBD, consisting primarily of UC and CD, is a chronic autoimmune (inflammatory) disease, which occurs in genetically susceptible individuals who are exposed to various environmental factors. The usual initial treatment of this spectrum of illnesses is medical therapy until treatment failure or until difficulties arise (192), after which surgical options are typically considered. During the course of their disease, many patients with CD and up to about one-third of patients with UC require some form of operative management (193, 194). Unfortunately, surgical therapy for IBD is associated with notable morbidity and mortality (195) and, thus, identifying clinical risk factors for colectomy in this population is of great importance. As mentioned hitherto, several studies have implicated CDI as presenting atypically in patients with IBD (78), and as being associated with an elevated risk for colectomy and death, predominantly in patients with UC (78, 80–83). However, unfortunately, more than a few studies published on this topic have based their diagnoses of UC and CDI solely on diagnostic coding data from medical databases, resulting in bias. Indeed, it would be imprudent and inappropriate to base imperative and life-changing clinical and surgical decisions on any single study published to date.

In this context, we conducted a systematic review of the literature in order to clarify the risk of colectomy among the subset of IBD patients with UC. Our study provides timely and invaluable clinical information, which will be relevant to patients with IBD and the practitioners that care for them at the bedside. Specifically, patients will have a better idea of what they may expect in

the course of their disease, and their physicians can plan their recommended courses of care with an understanding of the potential need for urgent surgical consultation. Furthermore, with a greater comprehension of the severe complications that the overlap between these two devastating dysbiotic disease processes can result in, an appropriate level of resources and funding can be solicited in order to bolster infection prevention & control measures and antimicrobial stewardship efforts, two major pillars in the battle towards reducing the risk of CDI and its complications.

QUESTIONS:

Primary: What is the risk of colectomy in patients with UC, who develop CDI?

Secondary: Are there any geographic differences in the risk of colectomy among UC patients who develop CDI? Is there a difference in the risk of colectomy among UC patients who develop CDI and undergo colectomy as inpatients as opposed to those who are followed-up and undergo colectomy later (post-discharge)?

3.2 Methods

Criteria for considering studies for this review:

Types of Studies

Original data presented in observational studies such as cohort, case-control and cross-sectional studies were included in the search strategy. Case reports/series, narrative reviews, pathogenesis articles, and randomized controlled trials (RCTs) were excluded.

Study Population

The study population included adult and pediatric patients with UC. All stages of UC and the diagnostic method or definition of UC, as provided by the authors, were considered for inclusion.

Studies where the study population included only patients with CD, IBD in general or atypical forms of IBD (e.g. collagenous colitis, lymphocytic colitis, diversion colitis, microscopic colitis, and indeterminate colitis) were excluded.

Exposure

The exposure of interest compared UC patients with or without CDI. The definition of CDI was based on the primary authors' definition of the presence or absence of CDI.

Outcome

The primary outcome of interest was colectomy at any time following the exposure of CDI. The identification of colectomy was based on the primary authors' definition of colectomy.

Search Methods for Identification of Studies

All aspects of this search strategy were developed, defined and conducted by two qualified investigators, Bayan Missaghi (BM) and Jenine Leal (JL). Dr. Missaghi is a licensed practicing infectious diseases specialist physician with credentials in infection prevention and control and is an MSc (Clinical Epidemiology) candidate. Mrs. Leal is an infection prevention and control epidemiologist and a PhD candidate with a major interest in CDI.

Electronic Searches

On October 19th, 2017 a comprehensive search for relevant articles using online medical databases, including Medline (Ovid), EMBASE (Excerpta Medica Database), and the Cochrane Library was performed. The search of online databases included all languages and was not limited by publication year or geography, but was limited to human studies. The first Boolean searches, within Medline, were conducted using the term "or" to explode (search by subject

heading) and map (search by keyword) following the MeSH headings "Clostridium difficile" or "enterocolitis, pseudomembranous." The second Boolean search was done using the term "or" to explode and map "Crohn Disease" or "ulcerative colitis" or "inflammatory bowel disease." These results were then combined using the Boolean term "and" to locate abstracts pertaining to both "Clostridium difficile" (and its derivatives and synonyms) and "ulcerative colitis" (and its derivatives, synonyms and encompassing terms—such as "inflammatory bowel disease"). Please see **Appendix B** for our medical database search strategy details. All titles and abstracts were extracted from the above-mentioned databases and managed using Endnote X8.1 (Thomson Reuters, 1988-2014).

Searching Other Resources

In addition, reference lists of all of the identified relevant publications were reviewed to ensure that all pertinent cited studies were identified in the electronic search. Study authors, for studies that did not report sufficient data, were contacted via email with respect to attempted acquisition of missing data.

3.3 Data collection and Analysis

Selection of studies

Using pre-specified eligibility criteria, BM and JL independently screened titles and abstracts for potential full text eligibility. If the reviewers deemed any title or abstract as potentially eligible, the articles were retrieved for full-text admissibility assessment. An abstract was considered for further review if it reported on original data from an original study (i.e. no review articles) and it reported on outcomes pertaining to IBD and CDI. All abstracts which reported original studies,

without clear or obvious IBD and CDI outcomes (but suggestive of these outcomes), were additionally pulled for detailed perusal to ensure that no applicable papers were missed.

Hence, the two reviewers independently assessed the eligibility of each full-text article according to pre-specified criteria. Studies for inclusion were required to report original data on the risk of colectomy among patients with UC who develop CDI relative to those without CDI. Any disagreement was resolved by consensus discussion between the reviewers. In the case of irresolvable disagreement between the reviewers, Dr. Gil Kaplan, an *a priori* third party adjudicator had been selected to assist in resolution and the consequent decision to include or exclude individual papers.

Data Extraction and Management

A data extraction spreadsheet was developed to collate information from each identified study. Please see **Appendix C** for details of our extracted study characteristics. The two reviewers independently extracted data from included studies on subjects comprising: study design, patient population, healthcare setting, number of patients, data sources, listed inclusion and exclusion criteria, sampling time frame, patients' age range, definition of CDI, definition and type of IBD, diagnostic method of IBD, outcomes measured, measures of association provided, definition of the measure of association and raw data to calculate the relative measure of association (i.e. odds ratios, risk ratios), as well as 95% confidence intervals.

Quality Assessment

The two reviewers (BM, JL) carefully assessed the quality of evidence of each study included. The criteria for assessing the quality of each study were adapted from the STROBE Statement and encompassed relevant criteria for assessing observational studies, inclusive of those used in the Newcastle Ottawa Quality Scale and the Downs and Black Checklist (196). These criteria included: assessment of the representativeness of the patient sample at baseline, whether the assessment of the exposure and outcome was objective and unbiased, whether the follow-up of the outcome was sufficient and complete and whether any adjustment for confounding was done.

Statistical analysis

The relative measure of association between CDI and colectomy among UC patients used was the odds ratio (OR) and its corresponding 95% confidence intervals (95% CI). This measure was extracted directly from the included studies or, when necessary, calculated using aggregate numbers provided in the studies. The statistical analyses were conducted using STATA/IC 13.1 (StataCorp LP, Texas USA, 1985-2013) and the commands "metan," "metafunnel," "metabias." The DerSimonian and Laird random-effects model was selected *a priori* to pool the ORs and their corresponding 95% CIs.

Assessment and investigation of heterogeneity

Heterogeneity was investigated using the I^2 statistic and the Chi^2 test (Cochran's Q Statistic). To explore possible explanations for heterogeneity, the following stratified analyses were planned *a priori*: region (i.e. North America vs. Eurasia), with an expected larger odds ratio among North American studies; setting (i.e. population-based studies vs. single, referral centres), with an expected larger odds ratio among single, referral centres; definition of CDI (i.e. laboratory and

symptoms vs. diagnostic code), with an expected larger odds ratio among studies using a laboratory and symptom-based definition; adjustment for confounding (i.e. <4 covariates vs. ≥ 4 covariates), with an expected larger odds ratio among studies with <4 covariates; and follow-up time for colectomy (i.e. in-hospital vs. longer follow-up times such as 30 days to 1 year), with an expected larger odds ratio among studies with a longer follow up time.

Assessment of publication bias

To evaluate the potential for publication bias and other small study effects, a funnel plot was inspected for visual evidence of asymmetry and then a Begg's test was conducted to assess the funnel plot asymmetry using an adjusted rank correlation.

3.4 Results

Description of studies

Results of the Search

A total of 9,322 original publications were identified, after the removal of duplicates, from our search of the online electronic databases Medline, EMBASE, and the Cochrane Library. Both reviewers appraised all 9,322 abstracts to determine relevance and eligibility. It was notably determined that BM and JL had an agreement of 99.86% at this initial stage of the review process. Thereafter, the reviewers read in detail 115 relevant articles that they had agreed to scrutinize and had perfect agreement on a decision to include 17 of them and to exclude 87 of them, resulting in 90% overall agreement (Cohen's kappa = 0.69) at this second stage of the review process, consistent with substantial agreement. The remaining 11 articles were the subject of further review and discussion and were eventually excluded. Disagreement—and final

decisions on whether or not individual articles should be included—was resolved by consensus discussion between the two reviewers.

Ultimately, of the 17 articles that were theoretically selected for inclusion (i.e. patient population being UC patients, CDI as the exposure variable, and colectomy as the outcome), 3 were excluded due to missing or insufficient data. This resulted in a total of 14 studies for inclusion in this systematic review and meta-analysis. Again, any disagreement between reviewers was resolved by consensus. Please refer to **Figure 1** for our study selection flow diagram.

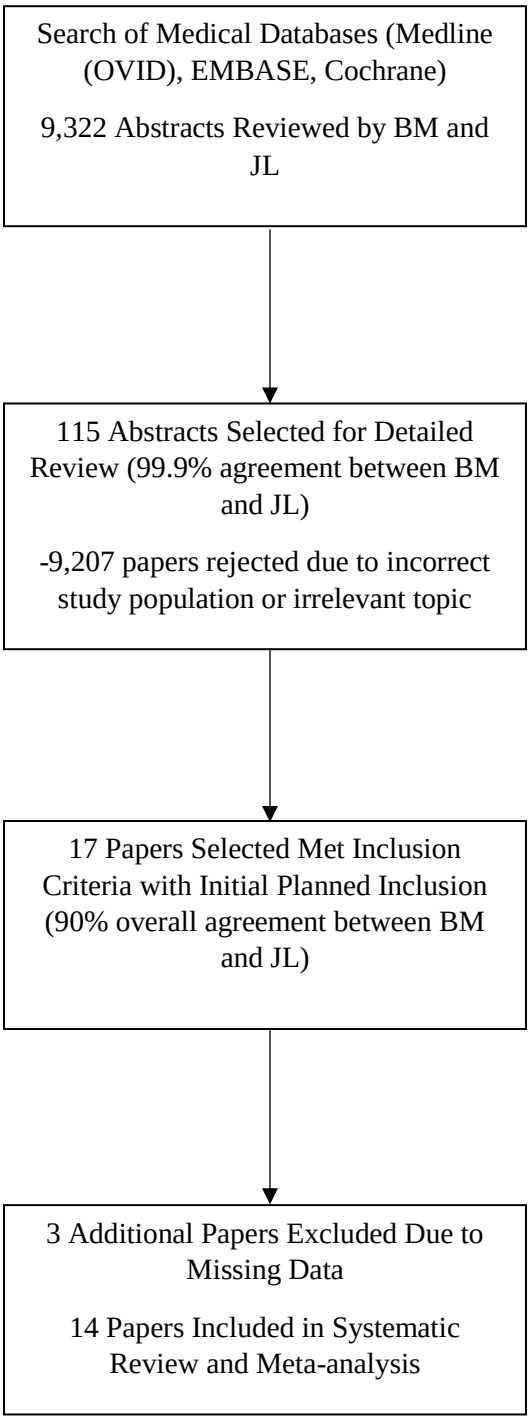

Figure 1. Study Selection Flow Diagram

Overview of Included Studies

The majority of the studies included were single-centered (9/14, 64%) and conducted in North America (9/14, 64%). Nine of the included studies were cohort studies (seven retrospective and two prospective), three studies were retrospective case-control studies and one was a survey. An additional study did not document a specific study type. Patients were included in these studies over a time period ranging between March 1980 and January 2016. All included studies assessed outcomes among adult patients, except for one which included pediatric patients (defined as < 18 years old) and one in which it was unclear what age range was included. **Table 3** outlines key characteristics of each study included. **Table 4** outlines key definitions of the patient population, of the exposure (CDI), outcomes measured and the crude and/or adjusted odds ratios and their corresponding 95% CIs. Crude ORs were calculated using available aggregate numbers in the studies, if the crude and/or adjusted ORs were not provided directly in the studies.

Table 3. Characteristics of studies reporting colectomy outcome among UC patients with and without CDI

Author	Publication Year	Study Design	Data Sources	Setting	Sampling Time Frame	Country	No. of Subjects	Age range (years)
Murthy et al.	2012	Retrospective, population-based, cohort study	Administrative databases and registries	Inpatient Acute care hospitals in Ontario, Canada	March 31, 2002 - March 31, 2008	Canada	2016	18+ years
Navaneethan et al.	2012	Historical, single-centre, cohort study	IBD Registry and electronic medical records	Inpatient, Outpatient IBD Center of Cleveland Clinic's Digestive Disease Institute	2002-2007	U.S.A.	146	No CDI: Mean 44.5 $\pm$ 15.7 years CDI: Mean 48.9 $\pm$ 18.0 years
Negron et al. (1)	2014	Retrospective, population-	Administrative databases and medical records	Inpatient	January 1, 2000 -	Canada	278	18+ years

		based, case-control study		Acute care hospitals in Calgary Zone, Alberta Canada	December 31, 2009			
Negron et al. (2)	2016	Retrospective, population-based cohort study	Administrative databases and registries	Inpatient Alberta province-wide hospitals	April 1, 2003 – March 31, 2010	Canada	1754	18+ years
Ott et al.	2011	Retrospective, single-centre, cohort study	Computer-based discharge registries, patient records	Inpatient Department of Internal Medicine, University of Regensburg; Tertiary referral centre	January 1, 2001 – June 30, 2008	Germany	128	Not documented
Kaneko et al.	2011	Retrospective, single-centre, survey	Not documented	Inpatient Yokohama City University Medical Centre; Referral centre	January 2006 - April 2009	Japan	41	14 – 82 years
Jodorkovsky et al.	2010	Historical, single-centre, cohort study	Administrative databases and electronic medical charts	Inpatient Mount Sinai Hospital, New York City	June 2004 - June 2005	U.S.A.	99	CDI Positive: Median 34 years CDI Negative: Median 38.4 years
Feuerstadt et al.	2012	Retrospective case-control study	Consecutive patients who had a *C. difficile* toxin assay	Inpatient Montefiore Medical Centre	2006 to 2008	U.S.A.	72	CDI+ IBD: 53.2 +/- 25.5 years; IBD alone: 51.0 +/- 21.7 years
Kroner et al.	2017	Retrospective cohort study	Nationwide Inpatient Sample (NIS), U.S.A.	Inpatient Nearly 1000 nonfederal short-stay hospitals; data from 22-40 states in the U.S.A.	2004 to 2013	U.S.A.	362,969 patients with UC	Mean age = 46 years
Morrison et al.	2015	Retrospective, population-based cohort study	Nationwide Inpatient Sample (NIS), U.S.A.	Inpatient Nearly 1000 nonfederal short stay hospitals; data from 22-40 states in the U.S.A.	2007 to 2011	U.S.A.	307,898 hospitalizations for UC	18 - 80 years
Smith et al.	2010	Cohort study	Case notes, hospital audit	Inpatient	January 2007 – 2009	United Kingdom	86	CDI: 55.5+/- 20.4 years

			database, in-hospital reporting system, discharge diagnoses	Not documented				No CDI: 26.8+/-16.8 years
Taylor et al.	2016	Prospective cohort study	IBD Inpatient Registry	Inpatient Alfred Health and Monash University IBD Unit	February 2015 – January 2016	Australia	15	CDI: 45+/-16.3 years No CDI: 40+/-13.4 years
Rolny et al.	1983	Not documented	Medical records	Inpatient Not documented	March 1980 – April 1981; May 1981 – December 1981	Sweden	40	Mean 48 years (range 18-82)
Kariv et al.	2011	Retrospective, Observational, Case-control Study	Cleveland Clinic IBD Registry	Inpatient, Outpatient IBD Centre of the Cleveland Clinic's Digestive Disease Institute	2000 – 2006	U.S.A.	78	Median age: 39 years (range 33-55)

Table 4. Characteristics, odds ratios and 95% confidence intervals of studies reporting colectomy outcome among UC patients with and without CDI

Author	**C. difficile Definition**	**Exposure Group**	**Comparator Group**	**Outcome Measured**	**Outcome among Exposed Group (UC+CDI) (n/N)**	**Outcome among Comparator Group (UC Only) (n/N)**	**Crude OR (95% CI)**	**Adjusted OR (95% CI)**
Murthy et al.	ICD-10 diagnosis code	Most responsible diagnosis of UC or MRD of *C. difficile* with co-morbid or secondary diagnosis of UC on hospital discharge abstract	Most responsible diagnosis of UC on hospital discharge abstract	In-hospital colectomy;* Adjusted 5-year risks of colectomy; Adjusted 5-year risks of colectomy among patients discharged from index hospitalization without undergoing colectomy	22/181	180/1835	1.27 (0.75 – 2.06)	0.91 (0.53 - 1.55)
Navaneethan et al.	Positive ELISA Test, Toxin A and/or B with concomitant	Concurrent diagnosis of UC and CDI	UC case with negative stool *C. difficile* test and no prior history of CDI	Colectomy within 1 year of initial *C. difficile* infection Colectomy within 3 months of initial *C. difficile* infection	16/45 3/45	10/101 9/101	5.02 (1.88 - 13.70) 0.73 (0.12 - 3.13)	10.0 (2.7 - 36.3)

	symptoms of colitis							
Negron et al. (1)	2-step approach EIA Test, Toxin A and B Sample considered positive if both tests positive	Diagnosis of *C.difficile* infection in hospital or 90 days before index admission among patients with a diagnosis of UC	UC case with negative *C. difficile* test in hospital or within 90 days before index admission	Emergent colectomy during index admission	11/17	91/261	3.42 (1.11 - 11.61)	3.39 (1.02 - 11.23)
Negron et al. (2)	ICD-10 diagnosis code	*Clostridium difficile* diagnosis any time between diagnosis of UC and colectomy	UC incident cohort without *Clostridium difficile* diagnosis	Colectomy by the presence of a colectomy procedure code	24/81	210/1673	2.93 (1.70 – 4.91)	
Ott et al.	ELISA Test, Toxin A and B and/or histological evidence with classic pseudomembrane formations.	Concurrent diagnosis of UC and CDI	Diagnosis of UC based on endoscopic, histological and radiological findings	Not defined	0/6	0/122	17.57 (0.196 – 1389.34); continuity correction of 1	n/a
Kaneko et al.	ELISA Test, Toxin A in gut lavage	Hospitalized patients with active UC and diagnosis of CDI	Hospitalized patients with active UC and negative investigation for CDI by gut lavage during colonoscopy	In-hospital colectomy	5/15 (33.3%)	6/26 (23.1%)	1.03 (0.41 – 2.63)	n/a
Jodorkovsky et al.	EIA Test, Toxin A and B; *C. difficile* stool culture; pathological evidence of *C. difficile*	Discharge diagnosis code for UC and *C. difficile* positive	Discharge diagnosis code of UC and *C. difficile* negative	(Emergent colectomy within 30 days of index admission) Emergent colectomy within 1 year of index admission	11/47 21/47	7/52 13/52	1.96 (0.62 - 6.58) 2.42 (0.95 - 6.23)	2.09 (0.72 - 6.10) 2.38 (1.01 - 5.60)
Feuerstadt et al.	*C. difficile* toxin assay positive	Biopsy-proven UC patients with CDI	Biopsy-proven UC patients without CDI	One year colectomy	18.20%	3.30%	5.8 (0.37 – 85.5)	n/a
Kroner et al.	Not documented	Patients with an ICD-9 code for a principle diagnosis of UC with CDI (unclear definition of CDI)	Patients with an ICD-9 code for a principle diagnosis of UC without CDI (unclear definition of CDI)	Colectomy	Not documented	Not documented	n/a	1.26 (1.05 – 1.53)
Morrison et al.	ICD-9 diagnosis code	UC patients with ICD-9 coding for CDI	UC patients without ICD-9 coding for CDI	Rates of in-hospital mortality and colectomy	477/19090	1733/288808	4.7 (4.2 – 5.1)	5.1 (4.5 – 5.8)

Smith et al.	Positive A/B toxin in standard stool sample testing	Joint diagnosis of CDAD and UC	UC alone	Colectomy	1/12	21/74	0.23 (0.005 – 1.79)	n/a
Taylor et al.	Stool culture plus EIA for Toxins A and B, not toxigenic culture or molecular testing	Acute severe UC with CDI	Acute severe UC without CDI	Inpatient colectomy	2/4	2/11	4.5 (0.18 – 94.73)	n/a
Rolny et al.	Fecal samples assayed for occurrence of *C. difficile* toxin	Patients with UC and CDI	Patients with UC and no CDI	Occurrence of *C. difficile* toxin in patients with symptomatic IBD and the extent of UC disease	½	0/38	2 (0.01 – 234.5); continuity correction of 1	n/a
Kariv et al.	Positive EIA test for stool *C. difficile* toxins A and/or B and infected if presented with concomitant symptoms of colitis	Patients with UC with positive results for *C. difficile* toxins A and B	Patients with UC with a negative stool *C. difficile* test and no prior history of CDI	Colectomy within 3 months of *C. difficile* testing	12/39	13/39	n/a	1.4 (0.49 – 3.7)

Quality Assessment

The quality of each study was assessed according to criteria adapted from the STROBE Statement as well as the Newcastle Ottawa Quality Scale and the Downs and Black Checklist. These detailed criteria and the reviewers' assessment of the quality of each study are displayed in **Table 5**. The green check mark indicates that the item was adequately addressed; the yellow caution mark indicates that the item was not clearly or completely addressed; and the red "x" mark indicates that the item was not addressed at all in the study. Most studies (11/14; 79%) did not provide adequate information on the methods used for measuring variables other than indicating the data sources from which variables were collected from. Four studies were considered high quality based on reporting on all or nearly all listed criteria (80, 197-199). On

the other hand, unfortunately, several studies did not include sufficiently long or complete follow-up (200-206). Of note, with the exception of the four "high quality" studies alluded to above, all the others were unclear with respect to whether a representative and well-balanced sample was selected at baseline.

Table 5. Assessment of study quality according to reviewers' judgment about each criteria. (N/A indicates that this criterion was not assessed in the study).

Study No	2644	4115	4613	5818	5896	5993	6018	6022	7802	8216	9323	3961	7093	4156
Author	**Feuerstadt et al.**	**Kaneko et al.**	**Kroner et al.**	**Morrison et al.**	**Murthy et al.**	**Navaneethan et al.**	**Negron et al.**	**Negron et al.**	**Smith et al.**	**Taylor et al.**	**Ott et al.**	**Jodorkovsky et al.**	**Rolny et al.**	**Kariv et al.**
Setting described?	✓	✗	✓	✓	✓	✓	✓	✓	✗	✓	✓	✗	✓	✓
Study Design reported?	✓	✗	✓	✗	✓	✓	✓	✓	✗	✗	✗	✓	✗	✓
Time referent reported?	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Eligibility criteria listed?	✗	✗	✓	✓	✓	⚠	✓	✓	✗	✗	✓	✓	✗	✓
Follow-up period indicated for outcome (cohort study)	N/A	✗	✗	✗	✓	✓	N/A	✓	✗	✗	✗	✓	✗	N/A
Cases/Controls defined (case-control study)	✓	N/A	N/A	N/A	N/A	N/A	✓	N/A	N/A	N/A	N/A	N/A	N/A	✓
Outcome clearly defined	✓	✗	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✗	✓
Confounders adjusted for	✓	✗	✓	✓	✓	✓	✓	✓	✓	✓	✗	✓	✗	✓
Data sources described	⚠	⚠	✓	✓	✓	✓	✓	✓	✓	✓	⚠	✓	⚠	✓
Methods of measurement of variables described	✗	✗	✗	✗	✗	✗	✓	✓	✗	✗	✗	✗	✗	✓

Biases addressed	⚠	✕	⚠	⚠	✓	⚠	✓	✓	⚠	⚠	⚠	✓	✕	✓
Study size described	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Representative and well-defined sample at baseline	⚠	⚠	⚠	⚠	✓	⚠	✓	✓	⚠	⚠	⚠	⚠	⚠	✓
Objective and unbiased assessment of outcome	✓	✕	✓	✓	✓	⚠	✓	✓	✓	✓	✕	⚠	⚠	✓
Follow-up sufficiently long and complete	✓	✕	✕	✕	✓	⚠	✓	✓	✕	✕	✕	✓	✕	✓
Adjustment for confounding	✓	✕	✓	✓	✓	✓	✓	✓	✓	✓	✕	✓	✕	✓

Association between CDI and Colectomy among UC Patients

The 14 included studies reported the association between CDI and colectomy among UC patients with varying follow-up times for their colectomy outcome. Four studies reported in-hospital colectomy (198, 200, 202, 204); one study reported colectomy within 30 days of the index admission (207); two studies reported colectomy within 3 months of the initial CDI (80, 82); four studies reported colectomy within one to five years of either the index admission or the initial CDI (81, 82, 207, 208); and five studies do not indicate the timeline of colectomy (199, 201, 203, 205, 206). In fact, one study did not define their outcome at all (205). To calculate the pooled OR and corresponding 95% CI, the adjusted ORs and 95% CIs reported in the studies were included and, if no adjusted ORs were provided, then the crude, calculated ORs and 95% CIs were included instead. The studies by Ott et al. (205) and by Rolny et al. (206) reported zero patients with the colectomy outcome. Therefore, for these two studies, a continuity correction of one was applied to all cells of the contingency table to calculate crude ORs and their corresponding 95% CIs.

The overall pooled result of the 14 studies suggests an increased odds of colectomy among UC patients with CDI compared to those without CDI (OR 2.3, 95% CI 1.3 – 4.1; random-effects). Statistically significant heterogeneity was detected for this comparison (heterogeneity chi^2 < 0.001) and according to the I^2, 92.9% of the total variability in the overall pooled result was likely due to heterogeneity across the studies and not due to chance alone. The forest plot for this association is shown in **Figure 2**.

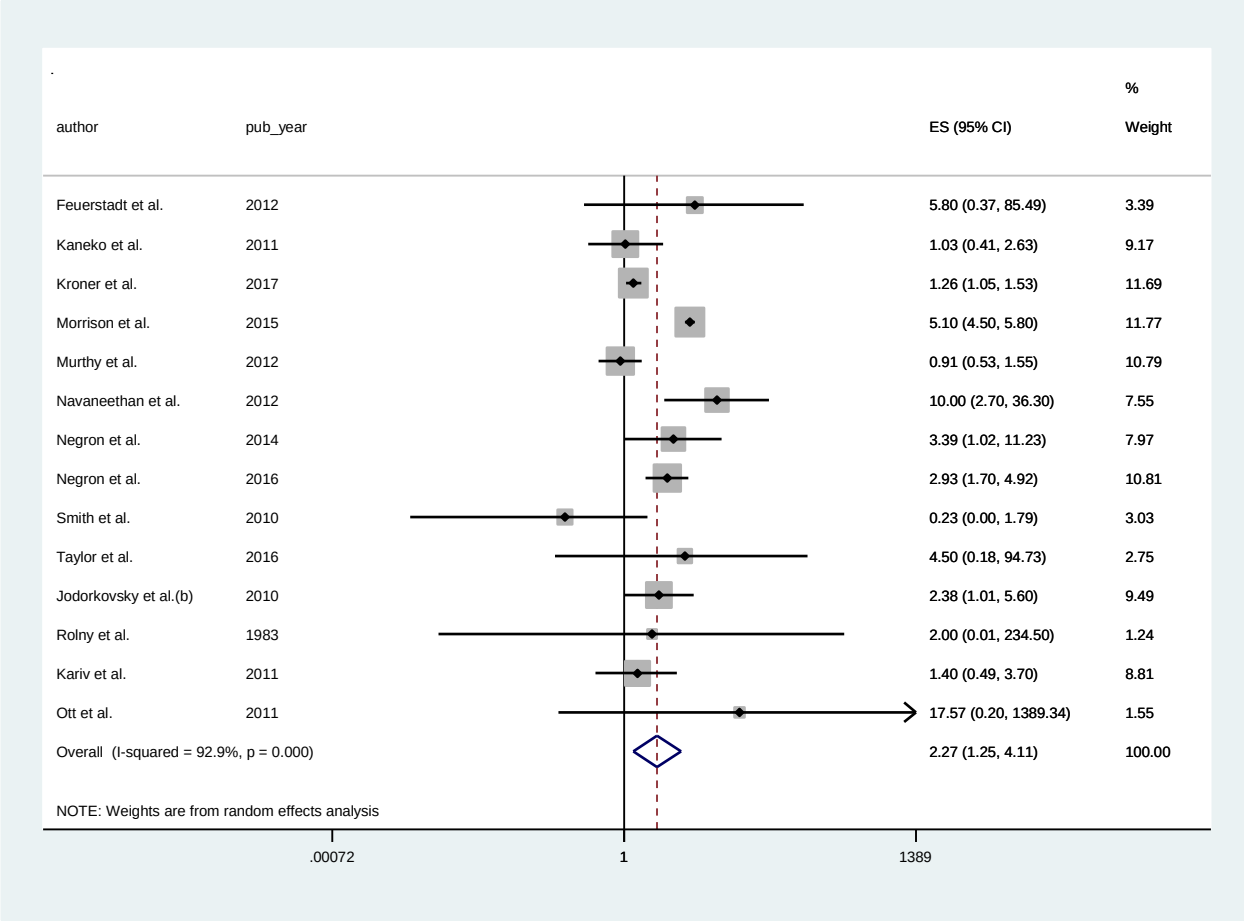

Figure 2. Forest plot of the association between CDI and colectomy among UC patients. Colectomy at any follow-up time.

Stratified Analysis

We considered the following *a priori* subgroups for the stratified analysis: region, setting, definition of CDI, follow-up time for colectomy outcome, and number of variables adjusted for in their analyses. **Table 6** lists the strata-specific ORs and 95% CIs and their assessments of heterogeneity. No subgroup resulted in statistically significant differences in the strata as all confidence intervals overlapped each other within each subgroup. Notably, studies that were conducted in a North American setting resulted in a significant pooled OR (2.5; 95% CI 1.3 – 4.9), though there was substantial heterogeneity across these studies (I^2 = 95.4%). Interestingly, studies conducted in Eurasia (i.e. Germany, Sweden, England and Japan) had virtually no calculated heterogeneity across studies (I^2=0.0%), though they did not result in a significant pooled OR (1.0; 95% CI 0.4 – 2.4). This suggests that there was little variability between these studies and that the variation seen may be due to chance alone. Moreover, studies that used a single-centre gave rise to a significant pooled OR (2.2; 95% CI 1.1 – 4.4) with a relatively small amount of heterogeneity (I^2 = 35.0%), and studies that used a laboratory and symptom-based definition of CDI resulted in a significant pooled OR (2.4; 95% CI 1.3 – 4.2), again with a relatively small amount of heterogeneity (I^2 = 30.1%). The same was true for studies with less than 4 variables adjusted for in the analysis, which resulted in a significant pooled OR (2.8; 95% CI 1.7 – 4.8) and these studies showed a moderate level of heterogeneity (I^2 = 65.1%). The largest pooled association (OR 3.1; 95% CI 1.3 – 7.4) between CDI and colectomy was observed among studies that looked at longer term outcomes (e.g. 30 days and 1 year), and this association was significant as the confidence intervals did not cross one.

Table 6. Stratified analysis of *a priori* subgroups for assessing heterogeneity in overall pooled estimate

Characteristic of Interest	Strata	Number of Studies	Pooled OR (95% CI)	I^2	p-value of Chi^2 statistic
Region	North America	9	2.5 (1.3 – 4.9)	95.4%	0.78
	Eurasia	4	1.0 (0.4 – 2.4)	0.0%	0.00
Setting	Single Centre	9	2.2 (1.1 – 4.4)	35.0%	0.32
	Population Based	5	2.2 (1.0 – 5.2)	97.6%	0.84
CDI Diagnosis	Laboratory	10	2.4 (1.3 – 4.2)	30.1%	0.24
	Diagnosis Code	3	2.4 (0.9 – 6.8)	95.0%	0.77
Number of Variables Adjusted for in Analysis	Less than 4	11	2.8 (1.7 – 4.8)	65.1%	0.32
	4 of More	3	1.3 (0.8 – 1.9)	50.1%	0.07
Follow-Up Time for Colectomy Outcome	Long Term	4	3.1 (1.3 – 7.4)	49.2%	0.37
	Hospital	10	2.0 (1.0 – 4.0)	94.9%	0.83

Assessment of Publication Bias

Funnel plots are typically used to assess the presence of publication bias, where studies showing a statistically significant effect of a treatment (or intervention) are more likely to be published and included in systematic reviews. Similarly, the quality of a study can influence the size of the treatment effect estimate with studies of lower quality typically showing larger effects. These biases tend to be more likely to affect smaller studies. The smaller the study, the larger the treatment effect needed for results to be statistically significant. On the other hand, larger studies

are more likely to be of higher quality and published even if the results are negative. Thus, bias in a systematic review can be assessed by reviewing the symmetry of a funnel plot. In the absence of bias, the plot will resemble a symmetrical inverted funnel. **Figure 3** is a funnel plot plotting each included study's odds ratio on a logarithmic scale versus each study's odds ratio's standard error (i.e. scatter plot of association with colectomy from each study against a measure of the study size). The plotting of the odds ratio on a logarithmic scale ensures that the effects of the same magnitude, but in opposite directions, are the same distance from 1.0. The y-axis is the standard error and it is used to emphasize the differences between studies of smaller size in which biases are most likely to occur. The y-axis is inverted so that as one goes up towards the point of the funnel, one finds the largest studies with the most certainty about the 95% CI around the overall pooled odds ratio, which is the centre line of the funnel plot.

Inspection of our funnel plot reveals some visual evidence of publication bias as there is some asymmetry observed. Most of the included studies in the analysis were presented as having low variance and located near the peak of the inverted funnel. Small studies, with higher variance and positive and negative associations between CDI and colectomy are missing from the analysis. In particular, there are no studies in the bottom right side of the plot, suggesting that smaller studies showing no statistically significant effects are missing from the meta-analysis (since they have not been published). These studies may impact the direction of the overall pooled OR for the association between CDI and colectomy among UC patients. However, of note, the Begg's test found no statistically significant evidence of publication bias ($p = 0.21$). The Begg's test assesses funnel plot asymmetry using an adjusted rank correlation and is reported when funnel plots are

created. Nevertheless, this test must be interpreted with caution when used in meta-analyses that include less than 25 studies, especially when the associated p-value is not significant.

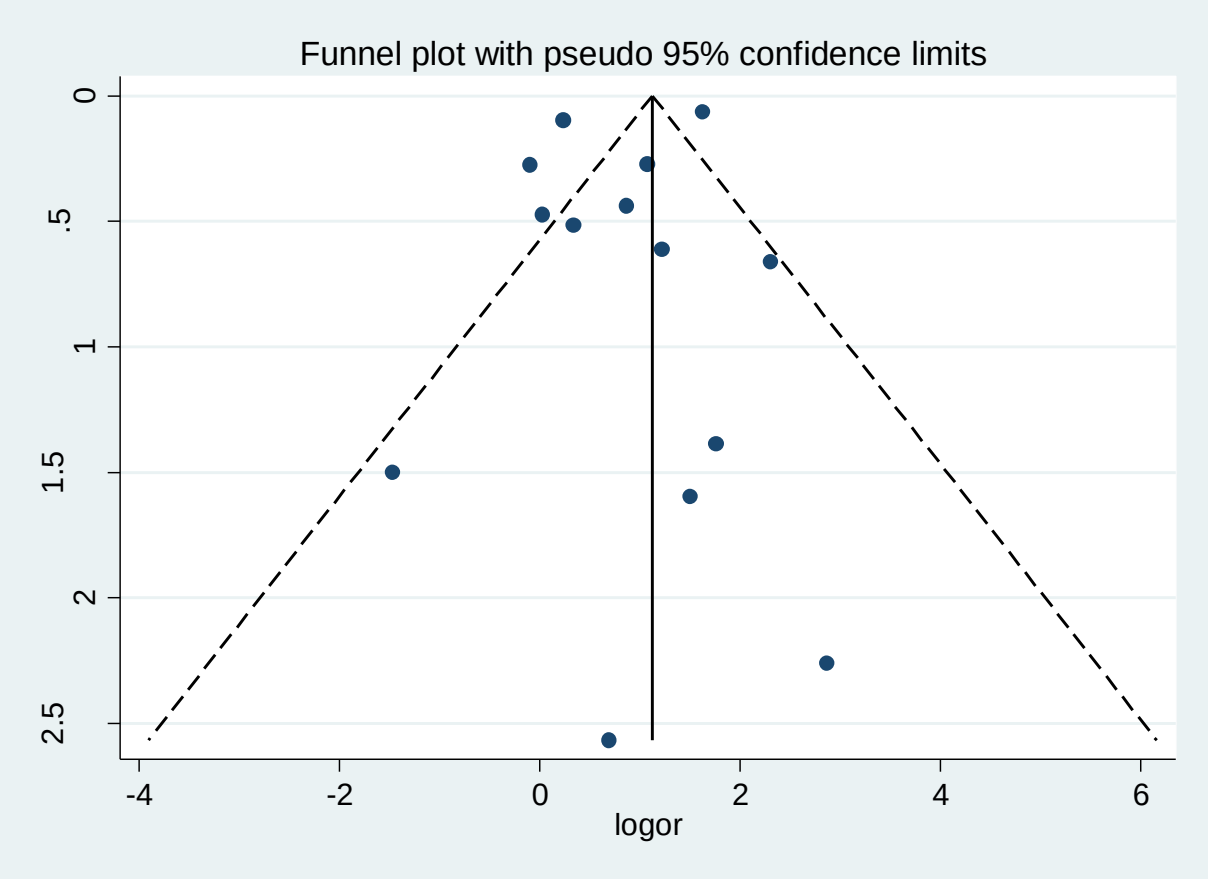

Figure 3. Funnel plot of the association between CDI and colectomy among UC patients.

3.5 Discussion

The overall pooled odds ratio of 2.3 (95% CI 1.3 – 4.1) suggests that UC patients with CDI are at an increased risk of colectomy relative to UC patients without CDI. Notably, this statement is consistent with our original hypothesis and has important implications for the clinical care of this population of patients. To state a few examples, UC patients may be provided with more accurate answers as to the expected possible course of their illness (should they develop CDI),

physicians may be made aware of potential complications with regards to their care of this group of patients and may consequently become more pro-active in seeking early surgical opinions and, importantly, funding may be solicited for prevention and management endeavors. It is notable that 11 of the 14 studies within our review did trend towards an elevated risk of colectomy, even if this risk was ultimately not determined to be significant. Importantly, we did choose a *random effects model* for our analysis, which assumes that the effect of CDI on the outcome for the individual studies vary around an overall mean association between CDI and colectomy among UC patients and that the association from the individual studies are normally distributed around this mean association. Due to the heterogeneity among studies, this model does tend to weight included studies more equally and, therefore, results in wider confidence intervals. Ultimately, we must acknowledge that with an $I^2 = 92.9\%$, our study contains a very high degree of heterogeneity that cannot be explained by chance or sampling error alone. Our Cochran's Q-statistic (Heterogeneity chi-squared) =182.2 with a p-value of < 0.001 lends further credence to this fact.

In an attempt to explain this heterogeneity, we conducted several stratified analyses, examining various study level characteristics as possible explanations for this variability, including differences by region (North America vs Eurasia), setting (single-centre vs population based), CDI diagnosis (laboratory vs diagnosis code), control of confounding variables (≥ 4 vs <4 confounders) and follow-up time to colectomy (long term vs. in-hospital colectomy). However, in each case, as confidence intervals overlapped, none of these stratified analyses could completely explain the heterogeneity amongst our studies. A possible explanation for this is that the relatively small number of studies that met our inclusion criteria, 14 in total, may have been

underpowered to demonstrate significant differences between them. Moreover, we acknowledge that this variability could be due to other original study-level characteristics that were not assessed by us directly.

In reference to study quality, we conducted our assessment using a number of parameters adapted from the STROBE statement as well as some tools from the Newcastle Ottawa Scale and the Downs and Black Checklist. Specifically, we primarily focused on the following 4 factors: a representative and well-defined sample at baseline, an objective and unbiased assessment of outcome, sufficiently long and complete follow-up, and adjustment for confounding. Although 4 of our included papers (80, 197-199) were quite comprehensive with regards to achieving excellence in each of these categories, several of our papers were vague or unclear with regards to one or more of these categories. Notably, 7 of the 14 articles that we ultimately included were deemed to have insufficiently long or complete follow-up, and as many as 10 of the 14 did not include data that would allow us to conclude that a representative and well-defined sample was determined at baseline. Please refer to **Table 5** for further details with respect to study quality. Unfortunately, as a meta-analysis is in many ways the sum of its parts, such study level shortcomings must inevitably be taken into consideration when drawing conclusions from our overall pooled results.

In this context, we acknowledge that our study had several limitations: some of these due to the overarching weaknesses inherent in meta-analysis methodology in general, and some due to the shortcomings of our included studies. It is generally well known that there are important nuances to performing a high quality meta-analysis and that, in some cases, a meta-analysis is not

appropriate and the results can be misleading. This is primarily due to several possible sources of bias.

First, in regards to the identification and selection of studies, three subtle, but important, problems can come into existence: publication bias and search bias in the identification phase in addition to selection bias during the selection phase of manuscripts (209). Briefly, publication bias speaks to the fact that a representative sample of articles on any topic is not likely to exist, as those studies yielding "positive" (i.e. beneficial) results are much more likely to be published than those yielding negative ones. Drug companies and other parties with financial interests, who are interested in gaining market share will be unlikely to encourage the publication of studies that show their products to be of no benefit, for example. This type of bias can best be combatted by making an extreme effort to identify all unpublished studies, an effort which we have made. The funnel plot in our analysis was used to assess publication bias and although the Begg's test was not statistically significant, visually there did appear to be some degree of publication bias. Most of the studies included in the analysis sat near the peak of the inverted funnel and there were a limited number of small studies included. Of note, the asymmetrical nature of the funnel plot in our study does not necessarily mean that publication bias is the only reason for heterogeneity among the studies. The asymmetry of the funnel plot may also be explained by other sources of bias, including differences in samples or underlying risk, poor methodological quality, inadequate analysis, inappropriate effect measure and/or chance. Of note, though we did not additionally review all of the recent conference proceedings or abstracts from major relevant organizations including, but not limited to the Infectious Diseases Society of America (IDSA), the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), the Society

for Healthcare Epidemiology of America (SHEA), the Canadian Association for Clinical Microbiology and Infectious Diseases (CACMID), the World Gastroenterology Organization, the Canadian Association of Gastroenterology (CAG), the American Gastroenterological Association, and the Congress of European Crohn's and Colitis Organization (ECCO), we did conduct a very comprehensive search of EMBASE and believe that most pertinent conference proceedings and abstracts were found using this database tool.

Another major source of bias, during the study selection phase of such a project, is selection bias, which typically occurs when investigators either do not have or do not follow strict objective criteria for inclusion and exclusion into their study. In our case, we certainly determined robust criteria *a priori* by which we objectively selected only those pertinent original studies that assessed our outcome of interest (colectomy) in our population of interest (UC patients), while exposed to our predictor variable of interest (CDI). However, given limitations of the publications we reviewed and gaps in information therein, not all of the information we sought from each study was readily available. We did mitigate this fact to some extent by contacting the study authors of otherwise relevant papers for unpublished figures and data, but were limited in the responses we received and the additional data that was made available to us. Nevertheless, we were able to obtain sufficient unpublished data to add one additional paper by Feuerstadt et al. (208) to our included list as a result of making such efforts.

As alluded to, there were many limitations to the 14 included studies in our systematic review. Half of these did not include adequate long-term follow-up of patients and the same number did not provide adjusted odds ratios for confounding variables. Many studies did not provide an ideal

amount of information on their colectomy outcome. Specifically, not all studies defined how the outcome was measured and whether patients underwent colectomy prior to the index CDI. Patients who had colectomy prior to the index CDI or who died prior to testing for CDI and were included could have resulted in a higher or lower association between CDI and colectomy than truly present, respectively. A few studies included UC patients that were tested for CDI and were negative as the comparator group, therefore excluding patients with UC who were not tested for CDI and leading to some patients and their outcomes being excluded from the analysis. Many studies were conducted at a single, referral centre which introduces referral bias where it may be likely that more severely ill UC patients were included in the study, thereby potentially increasing the risk of CDI and/or colectomy in that population. Moreover, there was little geographic representation with only a single study from Asia (Japan) (200), a single study from Oceania (Australia) (204) and three studies from Europe (one from England (203), one from Germany (205) and one from Sweden (206)). Thus, the external validity of this study is somewhat limited, given that the epidemiology of UC and CDI may vary between different regions and countries. Of note, only a single study that we reviewed that met our inclusion criteria included pediatric patients (200) (defined as < 18 years old). Finally, almost half of the studies we included were based on diagnostic codes from administrative databases without any corroboration with patient charts, medical records or laboratory confirmed results (80-81, 199, 201-202, 204). It is well known that a CDI-associated colectomy may not occur during an index admission, but rather during a subsequent hospitalization, and administrative data sets may miss an important association between surgery and CDI in such patients. Most of these papers cited this as a major weakness to their conclusions.

Therefore, this paper contributes interesting and valuable results to the existing body of literature and suggests that UC patients with CDI are at increased risk of colectomy relative to their counterparts without CDI. Future, better quality prospective studies, ideally with a population-based approach and with long term follow-up of outcomes, would be helpful to better evaluate this potential relationship under different circumstances. In addition, studies that focus on administrative databases could have preceding validation studies to ensure accuracy and correctness of such diagnoses. Lastly, papers that do chart reviews should have at least two data extractors to ensure that there is agreement in patient inclusion or exclusion.

3.6 Appendix B – Search Strategy Details

CDI IBD_2016
Medline 31 Aug, 2016 (updated with latest search Oct 19, 2017)

1. exp Clostridium difficile/
2. exp Enterocolitis, Pseudomembranous/
3. (CDAD or clostridium difficile or c difficile or c diff or CDI).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword]
4. exp Crohn Disease/
5. exp Inflammatory Bowel Diseases/
6. exp Colitis, Ulcerative/
7. (Crohn* or CD).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword]
8. (IBD or inflammatory bowel disease).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword]
9. (IBD or (inflammatory adj1 bowel)).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword]
10. (ulcerative colitis or UC or colitis).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword]
11. 1 or 2 or 3
12. 4 or 5 or 6 or 7 or 9 or 10
13. 11 and 12
14. limit 13 to (english language and yr="2014 -Current")
15. from 14 keep 1-619

CDI IBD_Cochrane
Cochrane 31 Aug, 2016 (updated with latest search Oct 19, 2017)

1. clostridium difficile.mp.
2. enterocolitis, pseudomembranous.mp.
3. (CDAD or clostridium difficile or c difficile or c diff or CDI).mp.
4. crohn disease.mp.
5. inflammatory bowel disease.mp.
6. colitis, ulcerative.mp.
7. (IBD or (inflammatory adj1 bowel)).mp.
8. (Crohn* or CD).mp.
9. (ulcerative colitis or UC or colitis).mp.
10. 1 or 2 or 3
11. 4 or 5 or 6 or 7 or 8 or 9
12. 10 and 11
13. from 12 keep 1-27

CDI IBD_Embase
Embase 31Aug, 2016 (updated with latest search October 19, 2017)

1. exp Peptoclostridium difficile/
2. exp pseudomembranous colitis/ or exp Clostridium difficile/
3. (CDAD or clostridium difficile or c difficile or c diff or CDI).mp.
4. exp Crohn disease/
5. exp inflammatory bowel disease/
6. exp ulcerative colitis/
7. (Crohn* or CD).mp.
8. ((IBD or inflammatory) adj1 bowel).mp.

9. (ulcerative colitis or UC or colitis).mp.
10. 1 or 2 or 3
11. 4 or 5 or 6 or 7 or 8 or 9
12. 10 and 11
13. limit 12 to english language

3.7 Appendix C – List of Extracted Study Characteristics

Study Design
Patient population
Healthcare Setting
Data Sources
Inclusion
Exclusion
Sampling time frame
Age Range of Population
Definition of CDI
Definition/Type of IBD
Diagnostic Method of IBD
Outcomes Measured
Measure of Association Provided
Definition of Measure of Association
Measure of Association
95% CI

CHAPTER 4: SUMMARIZING DISCUSSION

4.1 Conclusions

In summary, the rapid development of novel technologies over the past two decades, such as next generation deep sequencing techniques, has allowed us to begin to identify and characterize to an as of yet unparalleled degree the 10 to 100 trillion bacteria that live on and within the human body. Whereas in the past, most gut bacteria were considered generally unidentifiable due to the fact that they have fastidious growth requirements and are difficult to culture, we have now developed the tools to begin to better identify and comprehend their constitution. To date, we have learned that the "normal" microbiome varies by body site and may have a high degree of diversity depending on the populations being studied, leading to the concept that a healthy microbiome is challenging to define.

However, we have concurrently have come to understand that alterations in the typical or "normal" microbiome are definitely associated with various disease states, such IBD and CDI, as well as bacterial vaginosis (BV). In this well-studied condition that we have not hitherto addressed, the vaginal environment, which is usually dominated by *Lactobacillus spp*., becomes over-run by other bacterial species that are not typically present in this habitat, such as *Gardnerella vaginalis*. This is yet another example of a disease process that is primarily due to a disturbance in local bacterial homeostasis. As explored in Chapter 2 of this manuscript in the context of IBD in an analogous fashion, antibiotic exposure and infections have been demonstrated to result in the development of such conditions, and it is believed that the normal

flora of the female genitourinary tract play an important role in immune regulation and barrier function analogous to their role in the GIT (210).

As discussed in detail in Chapter 2, the worldwide obesity problem has reached epidemic levels, resulting in a sharp increase in the incidence of diabetes mellitus, metabolic syndrome, and resultant cardiovascular disease and other related problems. The traditional view of obesity that it is related to unbalanced energy intake, however, has been challenged by the potential contributory role(s) of changes to the intestinal flora, as a result of environmental exposures and influences (including diet), and the consequent impact on energy metabolism due to intestinal flora alterations, as clearly demonstrated in murine studies. One must wonder whether the "obese" phenotype in humans is, at least in part, secondary to a higher abundance of *Ruminococcaceae* and *Rickenellaceae*, given the well-documented association with leptin-resistance (147). In the context of well-described adaptations determined in response to varying diets in different regions, we must certainly respect the role of microbiome alterations and adaptations as a result of functional requirements. Indeed, it is fascinating to note that individuals in rural Africa and South America have gut microbiomes similar to those of herbivorous animals, whereas those of North American peoples are more consistent with those of carnivores.

Another pivotal focus of this paper has been the heavy impact of antimicrobial therapy on the microbiome. For approximately 70 years, a diversity of antimicrobials have been freely and liberally used in agriculture industry in animal feed, as growth promoters. Moreover, in many parts of the world and, in particular, in developing countries, these compounds may be acquired easily over-the-counter (OTC) or through inappropriate prescriptions, and a lack of appropriate stewardship has plagued humanity virtually since the initial discovery of antibiotics. With the knowledge that damage to the gut microbiome in the first year of life by such agents may result

in irreparable harm and lead to diseases such as atopy and IBD in individuals as well as growing resistance in both hospital and outpatient communities, physicians and medical researchers have a responsibility to combat this pervasive and high impact problem. As mentioned, one novel and creative way to do this is to develop scoring system(s) by which we measure the impact of antimicrobials on the microbiota and implement such approaches in the preclinical drug approval process to be certain that our drugs have the lowest possible microbiome damage profiles.

Moreover, as we have collectively learned more about the constitution of the microbiome (and the gut microbiome in particular) and its implications for both health and disease, we have naturally sought to use this knowledge in respect of developing therapeutic modalities and, as discussed in Chapter 2, some of the foremost potential applications of this concept include FMT, MET, probiotics and prebiotics. Indeed these therapeutic modalities are not only now a mainstay in the treatment of conditions such as CDI (and the prevention thereof) and IBD, but are also being trialed for conditions as diverse as Parkinson's disease, multiple sclerosis, metabolic syndrome, hay fever and autism (211). Treatment with SCFAs or the use of SCFA receptor agonists is another truly exciting potential therapeutic modality by which we may soon be able to strengthen intestinal barrier permeability and prime the immune system. Without a doubt, it would appear that the sky is the limit in respect to potential treatment implications of the microbiome.

Finally, in Chapter 3, we explored the relationship between UC and CDI, two important disease states each due in part to aberrancies in the microbiome, through a comprehensive systematic review and meta-analysis in terms of their combined impact on the rate of colectomy. CDI is an important and very deleterious infection that frequently affects IBD patients, especially those with UC, and which was postulated to result in a significantly increased rate of colectomy. Our

meta-analysis appears to confirm this fact, and provides very pertinent and relevant information to surgeons, gastrointestinal specialists and infectious disease specialists, who often need to collaborate closely to manage these often very ill patients. The possession of this knowledge allows us to better prognosticate our patients with UC, who are unfortunate enough to become afflicted with CDI, and helps us to dedicate appropriate funding and health care resources, working towards better (ideally "microbiome-based") treatment of such diseases and prevention thereof.

Ultimately, this dissertation demonstrates that which we have learned about the microbiome to date, how it is affected by dietary inputs and antimicrobials, its implications for health and disease and, importantly, how it may be manipulated as a therapeutic modality when homeostatic mechanisms fail and the balance is lost.

4.2 Future Directions

The launch of the Human Microbiome Project in 2007 was supported with ~$175 million investment from the NIH Common Fund (2007-2012) (212), and is a clear demonstration of the recognition of the microbiome's paramount importance to human health and wellbeing. Though a review of the medical literature over the past decade reveals an exponential growth of research and literature on this topic, it is believed that we are looking at the proverbial "tip of the ice berg" in respect to our current knowledge base and understanding of its constitution, mechanisms and implications.

Future studies will allow us to focus on microbial ecosystem functionality through the use of metagenomics to more deeply explore the microbiomes of clinical, environmental and

engineered habitats and will eventually result in the development of organism-based metabolic network models. Beyond merely characterizing the composition of the human microbiome, we will begin to discover and understand the underlying principles that govern the assembly, function and dynamics thereof. Concurrently, other researchers will seek to answer other fascinating related questions, such as the role that microbes have played in organismal evolution and the role that they may play in the future evolution of our species. Other scientists will continue to focus on the application of metabolomics to microbiota studies, learning more details about the metabolites of these organisms and how bacteria use them to communicate with one another and with the host. Naturally, this will result in an improved understanding of how we may be able to modulate specific microbes within a microbiota community. Of great importance, using our progressive understanding of the microbiome, there will be a revolutionary change in our integration of agricultural policies and practice, food production and dissemination, and we will likely formulate nutritional suggestions for people representing various ages, lifestyles, and states of health. As we move to the future, there will be amplified development within the field of synthetic biology, resulting in innovative and original opportunities for synthetic human microbiomes and/or their products.

Among other consequences, in the end, all of these studies will effectively allow us to gain a better, more detailed and more molecular understanding of the human microbiome and, ultimately, current dysbiotic conditions such as CDI, IBD and the intersection thereof. With this increased knowledge of the underlying cause(s) of these and other similar conditions, we will be able to formulate novel prevention and management strategies that are anticipated to become increasingly individualized and to include concomitant knowledge of each patient's genetic susceptibility profile as well as a clear perception of the environment in which they reside. A day

may exist in the not-too-distant future in which we are able to prevent many otherwise genetically susceptible individuals from becoming afflicted with these deplorable conditions in the first place. Moreover, those who do develop them will hopefully be treated in a much safer, rapid, and effective manner.

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APPENDIX A – COPYRIGHT PERMISSIONS

Bayan Missaghi

From: Pathogens Editorial Office <pathogens@mdpi.com>
Sent: Sunday, April 08, 2018 8:09 PM
To: Bayan Missaghi
Subject: Re: Permission to use Published Pathogens Article in Masters Thesis

Categories: Important

Dear Dr. Missaghi,

Pathogens is an Open Access journal publishing in gold open access model. All papers are published under the CC-BY licence where the authors retain the copyright for their work. So you can reuse the content and figures published in the Pathogens paper, provided that you cite them properly in your thesis.

In addition, we sincerely hope to you could publish with us again soon. Learn more about the recent publication at http://www.mdpi.com/journal/pathogens.

Kind regards,
Xiaoyan

On 2018/4/7 7:04, Bayan Missaghi wrote:
> To Whom It May Concern:
>
> My name is Bayan Missaghi and I am a practicing infectious diseases
> physician and the lead author of *Perturbation of the Human
> Microbiome as a Contributor to Inflammatory Bowel Disease* published
> in */Pathogens/* on June 30^th , 2014. I would like to include this
> manuscript in my Masters thesis, which will eventually be added to
> the institutional repository at the University of Calgary and the
> Library and Archives Canada.
>
> Further information can be found here:
>
> University of Calgary Theses Repository – The Vault
> http://theses.ucalgary.ca/Library and Archives Canada
> http://collectionscanada.gc.ca/obj/s4/f2/frm-nl59-2-e.pdf
>
> I have not been able to find anything on the MDPI website regarding
> formal acquisition of permission from /Pathogens/ for this purpose.
> However, given that this is the case with some other journals, I
> thought it would be prudent to contact /Pathogens/ to see if this is
> necessary and to subsequently follow the appropriate process.
>
> Thank-you, and please let me know if you have any questions or
> concerns. I will look forward to hearing you, preferably by April
> 15^th , 2018 (if at all possible). I do hope to collaborate with your
> journal again in the future.
>
> Kind Regards,
>
> Bayan
>
> Bayan Missaghi, MD, FRCPC, FACP, DTMH Clinical Assistant Professor of

Bayan Missaghi

From: Herman Barkema
Sent: Saturday, April 07, 2018 10:48 AM
To: Bayan Missaghi
Subject: FW: Permission to include Co-authored Paper in Masters Thesis

Categories: Finalized Publication, Important

"I, *Dr. Herman Barkema*, co-author of **Perturbation of the Human Microbiome as a Contributor to Inflammatory Bowel Disease** published in *Pathogens* on June 30th, 2014, grant Bayan Missaghi permission to include the listed manuscript(s) in his Masters thesis.

Best regards.

Herman

--

Herman W. Barkema DVM PhD FCAHS
Professor Epidemiology of Infectious Diseases
Faculty of Veterinary Medicine and Cumming School of Medicine (joint appointment)
NSERC Industrial Research Chair in Infectious Diseases of Dairy Cattle
Co-chair Clinical Research Unit (cru.ucalgary.ca)
University of Calgary

From: Subrata Ghosh
Date: Saturday, April 7, 2018 at 10:39 AM
To:

Subject: RE: Permission to include Co-authored Paper in Masters Thesis

"I, *Prof Subrata Ghosh* co-author of **Perturbation of the Human Microbiome as a Contributor to Inflammatory Bowel Disease** published in *Pathogens* on June 30th, 2014, grant Bayan Missaghi permission to include the listed manuscript(s) in his Masters thesis.

Subrata Ghosh
Professor of Translational Medicine
Director, Institute of Translational Medicine
University of Birmingham
United Kingdom

Bayan Missaghi

From:	Karen Madsen
Sent:	Sunday, April 08, 2018 10:52 AM
To:	Bayan Missaghi
Subject:	Re: Permission to include Co-authored Paper in Masters Thesis
Categories:	Finalized Publication, Important

"I, Karen Madsen, co-author of **Perturbation of the Human Microbiome as a Contributor to Inflammatory Bowel Disease**published in *Pathogens* on June 30th, 2014, grant Bayan Missaghi permission to include the listed manuscript(s) in his Masters thesis."

On Fri, Apr 6, 2018 at 4:48 PM, Bayan Missaghi wrote:

Dear All,

Good afternoon. I am writing to you because you are a co-author of **Perturbation of the Human Microbiome as a Contributor to Inflammatory Bowel Disease** published in *Pathogens* on June 30th, 2014. I would like to include this manuscript in my thesis, which will eventually be added to the institutional repository at the University of Calgary and the Library and Archives Canada. To be able to do so, I require your permission (if possible, by April 30th). Please reply to this email and write something like this (no signature necessary):

"I, *your name*, co-author of **Perturbation of the Human Microbiome as a Contributor to Inflammatory Bowel Disease** published in *Pathogens* on June 30th, 2014, grant Bayan Missaghi permission to include the listed manuscript(s) in his Masters thesis."

Further information can be found here:

University of Calgary Theses Repository – The Vault http://theses.ucalgary.ca/ Library and Archives Canada http://collectionscanada.gc.ca/obj/s4/f2/frm-nl59-2-e.pdf

Thank you, and please let me know if you have any further questions or concerns.

Sincerely,

Bayan

Bayan Missaghi, MD, FRCPC, FACP, DTMH, CTropMed
Clinical Assistant Professor of Medicine
University of Calgary