COMPARATIVE ANALYSIS OF HOST-BACTERIAL INTERACTIONS IN THE GASTROINTESTINAL TRACT

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COMPARATIVE ANALYSIS OF HOST-BACTERIAL INTERACTIONS IN THE GASTROINTESTINAL TRACT

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Technological advances in culture-independent methods using high throughput DNA sequencing have revolutionized our understanding of gastrointestinal (GI) microbiology. Our ability to now characterize the intestinal microbiome (IM) in incredible depth affords insight into the dynamic interrelationships of bacteria with the other pathomechanisms underlying inflammatory bowel diseases (IBD), i.e. genetics, immune dysregulation, and environmental triggers. This work explores these complex interactions in a murine model of Crohn's Disease (CD) and canine IBD.

In C57BL/6 mice, we reveal that *T. gondii* and indomethacin-induced ileitis induce a microbial shift termed 'dysbiosis,' comprising reduced microbial diversity and a global shift from >95% Gram+ (*Firmicutes*) to >95% Gram- species (*Proteobacteria*), which recapitulates the IM of ileal CD. Gram- proliferation also appears permissive to intramucosal invasion by the emerging pathogen, Adherent Invasive *E. coli* (AIEC). By manipulating genetic susceptibility to CD ($NOD2^{-/-}$) and severity of ileitis ($CCR2^{-/-}$ and anti-TNFα-mAb), we show that dysbiosis is the common end-point of acute ileitis, driven by inflammation rather than genotype or trigger. We postulate that failure to reverse dysbiosis stimulates chronic microbial-driven inflammation in CD.

Unlike other GI pathology, e.g. *Helicobacter*-induced gastric ulceration, the evidence for a specific pathogen in CD is controversial, though growing evidence implicates AIEC. Pathogenicity of AIEC is highlighted by their firm association with Granulomatous colitis (GC) of Boxer dogs. We strengthen the evidence for a direct causal relationship by documenting intramucosally invasive *E. coli* in 17 GC cases. Further, GC remission correlates with *E. coli* sensitivity to enrofloxacin, whereas antimicrobial-resistant *E. coli* are associated with poor outcome.

By genome-wide association study, we uncover for the first time parallels between GC and the colitis of human Chronic Granulomatous Disease (CGD), pointing to a potential role for AIEC in CGD pathogenesis.

In idiopathic canine IBD, we demonstrate dysbiosis characterized by *Bacteroidales* depletion and increased Gram- diversity. We highlight potentially distinguishing features of the IM in IBD that warrant further evaluation as potential predictors of treatment response.

In protein-losing enteropathy of Yorkshire Terriers, we document a severe, often fatal IBD, associated with sterile, multifocal crypt abscesses. Since we uncover no evidence of a bacterial etiology, we conclude that a familial morphogenetic disorder may be responsible.

BIOGRAPHICAL SKETCH

Melanie was born in the North-West of England. When asked on her third birthday what she was going to be when she grew up, she narrowed it down to: (i) a lollipop lady (nice old ladies who help school children to cross the road), (ii) an ice-cream van lady (a person that drives an old van playing loud music and selling ice creams called 99 Flakes) or (iii) a dog stroker. Many birthdays later she graduated from the Royal Veterinary College in London. After a year in small animal practice in aptly named Gravesend, she hotfooted it far North with a broken heart and a very big dog called Charlie. There she happened upon an unexpected vacancy for a small animal intern at the Royal (Dick) Vet School in Edinburgh. Great pubs, funny Scots, stunning scenery and good luck lead to a residency in small animal medicine with a gastroenterology focus. She obtained the Royal College of Veterinary Surgeons Diploma in Small Animal Medicine in 2005, and worked as an internist in referral practice for the next year. Although she was transiently wealthy she soon missed the challenge of academia and research. So when the opportunity arose to undertake clinical gastroenterology research with another funny Scot at Cornell University, she knew it was right up her street. And so she and Charlie flew to Ithaca in 2006, to undertake a PhD supervised by Dr Kenny Simpson. She obtained the European College of Veterinary Medicine Diploma in Companion Animal Medicine in 2009.

To my best friend and unsinkable ship Huda Trollope, with love and thanks.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AIEC	Adherent Invasive E. coli
ATG16L	Autophagy-related protein 16-1
bTEFAP	Bacterial tag-encoded FLX amplicon pyrosequencing
CACO-2	Colorectal adenocarcinoma cell line
CCR2	Chemokine receptor 2
CD	Crohn's Disease
CEACAM-6	Carcinoembryonic antigen-related cell adhesion molecule
CGD	Chronic granulomatous disease
DHR	Dihydrorhodamine 1,2,3
ELISA	Enzyme-linked immunosorbent assay
FISH	Fluorescence in situ hybridization
G14	*Giardia muris* post-infection day 14
G7	*Giardia muris* post-infection day 7
GC	Granulomatous colitis
GI	Gastrointestinal
GSD	German Shepherd Dog
GWAS	Genome wide association screen
HBSS	Hanks balanced salt solution
HDI	High dose indomethacin
IBD	Inflammatory bowel disease
IFNɣ	Interferon gamma
IGF2R	Insulin-like growth factor 2 receptor
IgG	Immunoglobulin G
IRGM	Immunity-related GTPase family M

LB	Luria Bertani
LDI	Low dose indomethacin
LPE	Lymphoplasmacytic enteritis
M6P	Mannose 6 phosphate
MAP	*Mycobacterium avium paratuberculosis*
MHC	Major histocompatability complex
MIC	Minimum inhibitory concentrations
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NCF2	Neutrophil cytosolic factor 2
NCF4	Neutrophil cytosolic factor 4
NF-ƙβ	Nuclear factor kappa beta
NOD	Nucleotide oligomerization domain
OCTN1	Organic cation transporter gene cluster 1
OmpC	Outer membrane protein C
pANCA	Peri-nuclear anti-neutrophil cytoplasmic antibodies
PAS	Periodic acid schiff
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLE	Protein losing enteropathy
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
RAPD	Random amplification of polymorphic DNA
SCWT	Soft Coated Wheaten Terrier
SNP	Single nucleotide polymorphism
T4	*Toxoplasma gondii* post-infection day 4
T8	*Toxoplasma gondii* post-infection day 8

TGF-β.	Tumor growth factor beta
TLR	Toll-like receptor
TMS	Trimethoprim-sulfa
TNFα	Tumor necrosis factor alpha
UC	Ulcerative colitis
uidA	*E. coli* gene for the beta-glucuronidase enzyme

CHAPTER 1

Introduction

It is incredible to think that we are all, at our core, essentially mobile incubators of a very diverse and proliferative bacterial 'soup,' housed within around 25ft of intestine. The gastrointestinal (GI) tract deploys multiple defense mechanisms to fight these potential intruders, including epithelial cell integrity, rapid cell turnover, autophagy, and the mucosal immune system. The studies herein are focused on evaluation of host-bacterial interactions in the GI tract, with particular reference to inflammatory bowel diseases (IBD) in people, domestic dogs, and mouse models. IBD is by far the most common cause of persistent GI symptoms in people and animals, and is characterized by chronic, idiopathic intestinal inflammation and varying degrees of debilitation, extraintestinal complications, and even death. The two main types of IBD affecting people are Crohn's Disease (CD), occurring principally in the ileum and colon, and ulcerative colitis (UC), which is restricted to the colon. In dogs and cats, lymphoplasmacytic enteritis (LPE) is the principal type of IBD, apart from the Boxer dog, which is affected by a breed-specific IBD, 'Granulomatous Colitis' (GC). The cause of IBD is widely accepted to involve the entanglement of multiple factors: the intestinal microbiome, genetic susceptibility, altered immune response, and environmental triggers of mucosal damage (Figure 1.1). The focus of this research is to enhance our understanding of these interrelationships in order to inform disease management, and facilitate the implementation of new targeted therapies.

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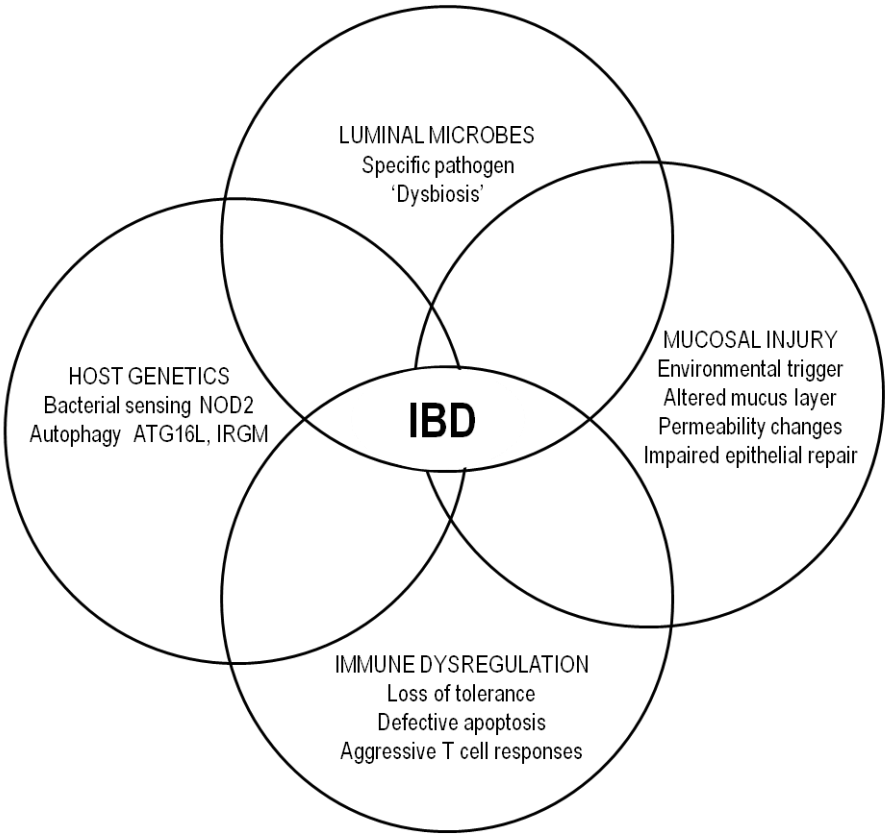

Figure 1.1: Overview of IBD pathogenesis.The etiology of IBD is multifactorial, involving luminal microbes, environmental factors, host genetics and immune dysfunction. The complex interrelationships between these factors are very poorly understood.

- **The GI Tract has a diverse indigenous flora**

The GI tract, in its simplest terms, is a long, continuous tube made from 4 layers of tissue: the mucosa, the submucosa, the muscularis and the serosa. Beginning at the mouth and extending through the thoracic and abdominal cavities, the main regions are the oral cavity, esophagus, stomach, small intestine (duodenum, jejunum and ileum), and large intestine (cecum, colon, rectum, anus). Each region provides a different set of environmental

conditions for potential microbial colonizers and has, therefore, a distinctive microbiota. The oral cavity contains around 10^{10} bacteria, comprising over 500 bacterial species. The stomach is a relatively hostile environment for bacteria, owing to its acidity and proteolytic enzymes, which are an essential barrier to the continuous ingestion of environmental bacteria. However, even in these conditions small numbers of bacteria can survive, most notably Gram-negative, spiral shaped *Helicobacter* organisms. These are important inducers of gastric inflammation, ulcers and cancer in people but their pathogenicity in dogs is controversial.[1] Gastric defenses are so effective that the duodenum and jejunum contain few bacteria in health, with aspirates of luminal fluid containing approximately 10^3 cultivable organisms/ml in most individuals, dominated by *Streptococci*, *Lactobacilli*, and anaerobic *Bacteroides*.[2] Further along the jejunum and into the ileum, bacterial populations begin to increase, and at the ileocecal junction they reach levels of 10^6 to 10^8 cultivable organisms/ml, with >99% composed of species within 4 bacterial divisions: *Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria*.[3] Yet higher numbers of bacteria with enormous diversity inhabit the colon, with concentrations of 10^9 to 10^{11} organisms per gram of luminal content. This includes a massive array of bacteria (>400 species), belonging to anaerobic genera such as *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Peptostreptococcus*, *Fusobacterium* and *Clostridium.*[2]

Each of us houses a unique population of microbiota that fluctuates according to environment, genetics, and disease.[4-12] However, until recently it has been very difficult to evaluate in depth the diversity of the intestinal microbiota, since only ~20% of species are cultivable. Owing to recent

advances in molecular methodology, culture-independent metagenomic surveys have revealed unprecedented microbial biodiversity.[13] Up to 40,000 bacterial species are estimated to comprise the collective GI microbiota, most of which have not been characterized by culture.[13] We are now able to study highly diverse microbial populations en masse without the need for culture, and through this methodology the genomes of entire populations of microorganisms can be studied in parallel.[13] Tools such as 16S rDNA clone libraries, pyrosequencing, quantitative PCR, and fluorescence in situ hybridization provide a deep description of the structure, function, and dynamics of microbial communities, and in turn be used to monitor health and disease.

- **Mucosal defenses**

The GI mucosa is constantly exposed to a vast array of endogenous microbiota and other antigenic material. This is essential for the development of both the digestive tract itself and our immune system, including GI lymphoid tissue such as Peyer's patches, and the production of immunoglobulin A.[14] At the same time, defending the integrity of the mucosal barrier from invasion by this vast and diverse array of co-inhabitants is as vital a function as absorbance of nutrients and fluids. The first frontier in mucosal defense is provided by anatomical barriers that physically halt penetration of bacteria, i.e. the intact intestinal epithelium and the integrity of epithelial tight junctions and intercellular junctions.[15,16] Specialized cells called Paneth cells are interspersed amongst epithelial cells to enhance protection against microbes and promote epithelial repair by secretion of antimicrobial peptides, e.g. defensins. Defective expression of α-defensin in Paneth cells has been

associated with ileal CD.[17] Goblet cells are another type of specialized cell that regulate production of the protective mucus layer and other epithelial repair factors, e.g. trefoil peptides and mucins.[18] Other antimicrobial agents include the cathelicidins, bactericidal/permeability increasing protein, and chemokines.[19]

The epithelium is in constant communication with an underlying network of innate and adaptive immune cells involved in sustaining normal mucosal homeostasis and mediating pathogen-specific responses.[15] The innate arm of the immune system provides an initial rapid response to microbes, mediated by epithelial cells, dendritic cells and macrophages that perform microbial sensing through pattern recognition receptors (PRRs) such as toll like receptors (TLRs) and nucleotide oligomerization domain (NOD).[20] Mutations in the gene encoding NOD2 are associated with development of CD.[21,22] Continual sampling of microbial antigens is essential for mucosal homeostasis and maintaining immune tolerance, and is performed by M cells of the epithelium, and by dendritic cells. In the adaptive immune response, dendritic cells present antigens to $CD4^+$ T cells, which carry out effector functions and initiate development of immunologic memory.[23,24]

Thus, intestinal health and repair depend on the interplay of an impermeable epithelial layer, secretion of antimicrobial peptides, repair of epithelial defects, and killing of translocating/invading bacteria. Defects in any one of these processes can result in increased microbial antigenic exposure, pathogenic T cell responses and chronic intestinal inflammation.[25]

- **Microbes induce intestinal inflammation**

Intestinal homeostasis involves the constant crosstalk between the gut microbiota and the host, in a mutualistic relationship.[26] The host immune system maintains tolerance towards the gut microbiota and the resident bacteria modulate the immune response to remain tolerated. When this dialogue becomes defective, inappropriate immune activation and inflammation ensue.[10,15,16,27-33] There is now a general consensus that IBD arises from an abnormal immune response to commensal flora. The evidence for this is abundant and arises from clinical trials, experimental rodent models, and translational and basic research.[10,15,16]

Clinical evidence:

* Therapeutic responses in UC, CD and pouchitis to fecal stream diversion, antibiotics, pre- and pro-biotics.[34-36]
* Serologic responses to microbial antigens in CD and UC, e.g. *Bacteroides*, *Pseudomonas*, *E. coli* OmpC, *Mycobacteria*, peri-nuclear anti-neutrophil cytoplasmic antibodies (pANCA).[37-39]
* Preferential detection of bacterial DNA in intestinal biopsies.[40,41]

Evidence from animal models:

* Gnotobiotic mice monoassociated with single commensal species (*E. coli* or *Enterococcus faecalis*) develop colitis.[42]
* Commensal bacteria are required for chronic inflammation in most rodent models.[43]
* Mice susceptible to inflammation in the germ-free state (IL-2 and IL-10 deficient, and Samp-Yit mice) have more aggressive inflammation when colonized by normal enteric bacteria.[43, 44]

- Colitis in primates decreased in colonic loops that were excluded from the fecal stream, and recurred following reperfusion of fecal contents.[45]
- Antibiotics inhibit experimental inflammation in multiple rodent models.[43]

Evidence from research:

- Mucosally associated bacteria, especially *Enterobacteriaceae* and *Bacteroides* are increased in IBD tissues.[46,47]
- 'Dysbiosis': altered ratio of harmful versus protective bacteria[3, 48, 49]
- Association of ileal CD with[49] Adherent Invasive *E. coli*.[50, 51]

- **Evidence for microbial involvement in Crohn's Disease**

It has long been recognized that bacteria are essential to the development of CD, but the precise mechanisms involved are the subject of much debate.[3] Traditionally, CD was widely thought to be associated with altered tolerance of commensal flora, leading to an NF-kβ mediated pro-inflammatory response.[52] However, recent advances in the aforementioned culture-independent metagenomic technology have demonstrated global changes in the bacterial microenvironment of CD. Microbial imbalances, termed 'dysbiosis,' suggest a significant contributory role of the commensal flora in disease pathogenesis, associated with loss of beneficial/regulatory bacteria, and enrichment of more pathogenic species.

1. Specific pathogen theory:

a. Mycobacterium avium paratuberculosis (MAP): the single pathogen most often implicated as a causative agent of CD is MAP.[53,54] This is based on the striking similarity of Crohn's disease with Johne's disease of cattle, characterized by severe transmural granulomatous inflammation. There have

however been conflicting reports regarding MAP DNA positivity from Crohn's biopsies, with some studies reporting positive findings and others not. Thus, a link between MAP and CD remains much debated.[55]

b. Adherent Invasive E. coli (AIEC): *E. coli* is the predominant cultivable aerobic Gram-negative species of the normal intestinal flora, and is increasingly implicated in CD. *E. coli* are abnormally prevalent in the ileal mucosa of CD patients, representing over 50% of the total number of bacteria[40] Using immunocytochemistry*, E. coli* antigen was demonstrated in 69% of diseased intestinal resection specimens from CD patients,[56] versus 6% of ileal controls,[51] and has been recovered from 18-31% of resected lymph nodes. Their enrichment in ileal mucosa has been correlated with the severity of disease, and AIEC have been found to invade inflamed ileum.[49] Ability of AIEC strains to invade, persist and replicate in intestinal epithelial cells and intracellularly within macrophages implies a role specifically in ileal versus colonic CD. Isolated from a CD patient, reference AIEC strain LF82 replicates rapidly within active macrophage phagolysosomes. Presence of *E. coli* in macrophages within the lamina propria, and in 80% of micro-dissected granulomas from CD patients provides strong support for a pathogenic role.[57] CD-associated AIEC strains express type I pili for adhesion to carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM-6), expression of which is upregulated by TNF-α secretion.[58] Thus, AIEC appears to promote its own colonization by inducing secretion of large amounts of TNF-α.[59] AIEC are unlike enteropathogenic *E. coli* spp. and do not possess toxin producing genes; they express virulence genes that mediate epithelial adherence and iron acquisition.[49] From a comparative aspect, they resemble

the invasive *E. coli* strains that have been indisputably implicated in the pathogenesis of Boxer dog GC.[28]

2. Dysbiosis theory:

Numerous investigators have demonstrated imbalances in the mucosa-associated microbiota in CD, UC, and pouchitis.[5,13,14,46,60] The picture emerging is that inflammation is associated with (a) decreased microbial diversity, (b) depletion of Gram-positive flora, predominantly the *Firmicutes*, including *Clostridia*, and protective *Faecalibacterium prautznitzii*, and (c) enrichment with potentially harmful Gram-negative members of the *Enterobacteriaceae*, including *E. coli.*[49,60,61]

The major unresolved question is whether dysbiosis represents primary versus secondary change. In support of the latter are the responses of CD patients to steroids and other immunosuppressive therapies,[62,63] and documentation of a common pattern of dysbiosis in rodent models, irrespective of intestinal injury.[64] Conversely, transfer of inflammation-associated luminal content induces pathology in healthy mice,[65] and potentiates dysbiosis in genetically susceptible mice, suggesting a primary pathogenic role.[66] The general consensus in studies of dysbiosis is that *E. coli* number is increased in the mucosa-associated microbiota of CD. *Bacteroides* however, have been reported to be enriched and depleted in separate studies.[60,67,68] The manner in which dysbiosis is harmful to the host is not clear, but it is likely to be associated with metabolic consequences to the epithelium of the alteration in microbial composition. For example, *Clostridia* produce butyrate and other short chain fatty acid molecules that are utilized as fuel by

colonic epithelial cells. Deficiency in butyrate, and other microbial-origin epithelial nutrients may be linked to pathogenesis in CD.

- **Host genetic regulation of the microbiome**

The other major players in IBD pathogenesis are host genetics and environmental influences, and their relative contributions are poorly understood. Studies in twins have provided powerful insights into IBD pathogenesis, and a disease concordance rate of approximately 50% in monozygotic twins highlights that genetics is a major contributing factor in CD risk.[69,70] However, an equal discordance rate in monozygotic twins[70] indicates the importance of environmental factors, particularly the microbiota.[5,6,71] By examining the intestinal microbiota in twin pairs divergent or concordant for CD or UC, Willing et al determined that the host genetics is not solely responsible for differences in the gut microbiota. Discordant twins segregated with respect to specific bacterial populations in accordance with disease phenotype rather than their twin. This suggests that bacterial composition is associated with disease activity moreso than host genotype. Nevertheless, there are well established genetic susceptibilities to CD that have been identified by genome wide association screen (GWAS), with commonality of dysfunctional bacterial-host interfacing, e.g. NOD2, autophagy (ATG16L, IRGM), and bacterial killing (NCF4). Both NOD2 and ATG16L have been associated with dysbiosis in CD patients.[66]

* NOD2 encodes an intracellular sensor of the bacterial wall peptidoglycan, muramyl dipeptide. Approximately 30% of CD patients have a NOD2 polymorphism, which if homozygous, confers greater risk

of CD by a factor of 11 to 27.[72] It is assumed that this is associated with defective bacterial sensing or killing, but is not well understood.[16,73]

- Autophagy genes ATG16L and IRGM are involved in clearing intracellular components, including microbes.[74] CD-associated polymorphisms in these genes implicate defective innate immune responses.
- NCF4 was recently associated with CD in a GWAS.[75] It encodes a subunit of NADPH oxidase, involved in phagocytosis and production of reactive oxygen species that are essential for bacterial killing.

- **Defective immunoregulation in Crohn's disease**

Mucosal $CD4^+$ lymphocytes are thought to play central roles in both the induction and persistence of chronic inflammation by producing proinflammatory cytokines.[76] In simplistic terms, CD exhibits a T helper 1 (Th1) immune response, triggered by microbes that stimulate production of IFNɣ and IL12p40. Another $CD4^+$ T cell lineage (Th17) has been more recently recognized, believed to arise from the same precursor population as regulatory T cells, but little is known about how these T-cell subsets interact under chronic inflammatory conditions. Th17 development is promoted by IL23, and polymorphisms in the IL23 receptor (IL23R) are highly associated with CD.[76] T cell tolerance in the intestine is mediated by inhibitory cytokines IL10 and TGF-β.[10,15]

- **Canine Inflammatory Bowel Disease**

Idiopathic IBD is the most common cause of chronic GI signs in dogs.[29,77,78] Clinical signs vary depending on the region of the GI tract affected, but

diarrhea, vomiting and weight loss occur most frequently.[29] The mechanisms responsible for the development of IBD are poorly understood, and consequently treatment has traditionally been based upon empirical combinations of diet, antimicrobials, and immunosuppression.[78-80] In dogs, IBD is classified according to histopathological characteristics, i.e. lymphocytic-plasmacytic, eosinophilic, histiocytic.[81,82]

The development of chronic mucosal inflammation in dogs, as in people, is widely thought to be multifactorial, and associated with environmental factors, genetic factors, immunoregulatory defects, and luminal microbes.[10,29,77] It is generally thought that altered immunological tolerance following an acutely damaging 'trigger' results in an overly aggressive immune response to luminal antigens such as diet and microbes.[10,16] In dogs, dietary ingredients have often been cited as a potential environmental trigger, but the evidence to support this is largely anecdotal and there are no studies that conclusively show a direct link.[83]

The study of canine IBD suggests that interplay between genetic factors and enteric bacteria are crucial for disease development, owing to abnormal host responses directed against the commensal microbiota. In support of this notion, several dog breeds are recognized as being predisposed to specific forms of IBD, including immunoproliferative enteropathy in Basenjis, protein-losing nephropathy (PLE) in Soft-Coated Wheaten Terriers, and GC in Boxers.[84-87] Genetic susceptibilities in canine IBD are largely unexplored, though a genetic basis is suspected in predisposed breeds such as the Lundehund, Soft-Coated Wheaten Terrier, Basenji, German Shepherd Dog, and Irish Setter and Boxer dog.

Immunoregulatory defects, particularly reduced immunological tolerance to luminal microbes, are proposed to drive a chronic inflammatory response in canine IBD.[10,28,88-91] Circumstantial evidence to support this is provided by the observed clinical benefits of antibiotic treatment in dogs, as well as recent reports that prebiotics and probiotics may ameliorate IBD.[30,80] The strongest indication of microbial involvement in canine IBD is undoubtedly the discovery of invasive intramucosal *E. coli* in the colonic mucosa of Boxer dogs with GC.[28] Notably, the *E. coli* strains isolated from the ileum of CD patients and the colon of Boxer dogs are all AIEC strains (adherent invasive *E. coli*), sharing similar virulence gene profiles.[28,92]

German Shepherd Dogs (GSDs) show increased susceptibility to chronic enteropathy, typified by lymphocytic-plasmacytic inflammation and clinical responses to antibiotics, diet, or immunosuppression.[77,93,94] Recent studies in GSDs with IBD using have identifed evidence of dysbiosis in association with aberrant expression of the PRRs, toll-like receptor (TLR) 4 and TLR5, respectively.[95] Additionally, multiple SNPs were identified in the NOD2 gene of both IBD-affected GSD and IBD-affected non-GSD breeds, suggesting that NOD2 mutations contribute to chronic mucosal inflammation in heterogeneous canine populations. (Kathrani abstract, ACVIM 2009). Additional evidence of dysfunctional innate immunity in canine IBD includes the observation that TLR2, 4, and 9 were up-regulated in duodenal and colonic mucosa of IBD affected dogs relative to controls.[88] A recent study has also shown NF-κβ activation in lamina propria lymphocytes of dogs with IBD.[96]

Emerging culture-independent studies indicate that imbalances in the intestinal microbiome are a consistent feature of canine IBD.[32,33] In separate

molecular studies, the diseased intestines of dogs with IBD were shown to be enriched with members of the families *Enterobacteriaceae* and *Clostridiaceae*.[32,33] A pathogenic role for luminal bacteria in canine IBD is also suggested by treatment responses to the antimicrobials metronidazole and tylosin.[31,97] Thus, a growing body of evidence points to the importance of intestinal dysbiosis in canine IBD.

- **Granulomatous colitis of Boxer dogs**

Granulomatous colitis (GC) is a relatively rare type of IBD, seen predominantly in Boxer dogs under 4 years of age.[86, 98-100] There are sporadic reports of GC in other dog breeds,[101] particularly young French Bulldogs[102] (and author observation). Affected dogs typically present with signs of colitis, hematochezia and weight loss, progressing to cachexia in severe cases.[98,103-105] GC was first reported in the US in 1965 [98] and later emerged in Australia, Japan and Europe, becoming better known as 'Histiocytic Ulcerative Colitis' (HUC). However, the original name as described by van Kruiningen appears more apt,[98] because it more accurately reflects the histopathological appearance of the inflammatory response, i.e. a mix of macrophages, lymphocytes and neutrophils, almost invariably reported by pathologists as 'granulomatous inflammation.' [86,99,105-109] Second, a histiocyte is a fixed tissue macrophage and the mucosa in GC is transiently packed with recruited macrophages that egress with successful treatment.[110]

The cytoplasm of macrophages in GC stains positive with period acid-Schiff (PAS), a unique and pathognomonic feature that is strikingly similar to Whipple's disease of people.[98] Whipple's disease is a rare systemic bacterial infection primarily affecting the small intestine. It is caused by the bacterium

Tropheryma whipplei, and diagnosed by finding PAS-positive macrophages in intestinal biopsies.[111,112] Because of this similarity, and following the occurrence of GC in 9 Boxer dogs from the same kennel, 6 of which responded to chloramphenicol treatment[98] an infectious etiology has long been suspected in GC. Thus, initial studies focused on the search for a GC-associated pathogen. Electron microscopic imaging of colon mucosa revealed occasional bacteria in 4 of 13 affected dogs, and abundant coccobacillary structures resembling *Chlamydia* within the macrophages of 5 dogs.[113] In a later report of GC, the isolation of a *Mycoplasma* spp. from the colon of 4/11 dogs and draining lymph nodes of 3/11 dogs raised the possibility of *Mycoplasma* as an etiologic agent. However, experimental inoculation of 8 week old Boxer puppies with the isolated *Mycoplasma* spp. did not induce colitis.[114]

With no definitive evidence for a specific pathogen, other authors suggested that the scant bacteria visualized within the superficial mucosa were opportune invaders of an inflamed and ulcerated mucosa.[84,103] A primary immune-mediated pathogenesis was presumed, and evaluation of the mucosal immune response in GC was undertaken using immunohistochemistry.[84,103] This revealed increased numbers of IgG^+ plasma cells, $CD3^+$ T cells, L1 cells and MHC class II cells, analogous to Ulcerative Colitis (UC) in people.[10] Until 2004 the mainstay of treatment for GC involved immunosuppression with agents such as corticosteroids and azathioprine, in combination with antibiotic therapy and dietary change.[99] Responses to

treatment were generally poor, frequently resulting in euthanasia. GC became considered an incurable immune-mediated disease.[84,100-103]

GCB and Invasive E. coli

The hunt for an infectious cause of GC was re-ignited by reports of long-term remission in dogs treated with enrofloxacin.[28,99,115,116.] The application of culture independent molecular methods, namely immunohistochemistry and fluorescence in situ hybridization (FISH), enabled the identification of mucosally invasive *E. coli*. Using a polyclonal *E. coli* antibody, immunoreactivity was documented in the lamina propria macrophages and regional lymph nodes of 10 affected dogs.[116] Immunostaining of colonic mucosa was also positive with antibodies against *Salmonella, Campylobacter* and *Lawsonia intracellularis*. Concurrent work using advanced molecular methods demonstrated the presence of metabolically active invasive *E coli* packed within colonic macrophages.[28] This was accomplished using FISH, a technique employing fluorescent molecules attached to oligonucleotide probes that hydridize to bacterial 16S ribosomal DNA. Fluorescent labeling enables clear visualization of bacterial morphology and spatial localization, even against a busy background of severe inflammation. In this study, FISH analysis in 13 GC cases was targeted by eubacterial 16S rDNA library construction generated from GC mucosa. The authors discovered intramucosal and macrophage invasion exclusively by *E. coli* in all cases evaluated.[28] GC-associated *E. coli* were shown to lack genes associated with virulence in diarrheagenic *E. coli*, and were able to invade epithelial cells and persist within macrophages.[28] This pathogen-like behavior is similar to the AIEC that are increasingly associated with CD.[40,51]

A direct causal role for *E. coli* in GC pathogenesis is supported by the correlation between clinical remission of disease and eradication of invasive *E. coli* using enrofloxacin.[28,110] A series of 7 dogs with histologically confirmed GC and intramucosal *E. coli* invasion on FISH were treated with enrofloxacin (7±3 mg/kg/day for 9.5±4 weeks), and re-evaluated by repeat histology and FISH.[110]. Long-term clinical remission coincided with the eradication of invasive *E. coli* in 4 dogs. In a relapsing case the *E. coli* was enrofloxacin resistant, and the animal was euthanized due to refractory disease. Interestingly, the PAS staining in this study remained positive for >6 months despite remission of clinical signs and eradication of invasive *E. coli*. The reasons for this are not clear, but it is important to note that complete histologic remission of disease appears to lag behind clinical improvement, a feature also reported in Whipple's disease.[117]

The importance of appropriate antimicrobial selection in the treatment of GC is demonstrated here in Chapter 3, in a prospective study of 14 GCB.[92] In this study, the *E. coli* isolates from 6/6 'complete responders' were enrofloxacin sensitive, whereas those from 4/4 'non-responders' and 2/4 'partial responders' were enrofloxacin resistant. Clinical response was directly influenced by susceptibility of *E. coli* to enrofloxacin ($p<0.01$).

Taken as a whole, this evidence indicates a 1:1 correlation between GCB and invasive *E. coli* in 32 cases collectively evaluated by FISH to date.[28 92,110] This discovery has transformed the diagnostic approach, therapy and prognosis of GC. The Boxer breed specificity and disease characteristics suggest an inherited autosomal recessive immune defect that impairs the elimination of invasive *E. coli,* which we explore in Chapter 4.

In summary, the commensal microbiota plays a pivotal role in maintaining intestinal health, and imbalances in the dominant floral groups are increasingly recognized to play a role in the development of IBD in people and animals. Our rapidly expanding knowledge in this area has been facilitated by the advancement of technology in metagenomics in recent years. Intestinal dysbiosis, characterized by reduced species diversity, depletion of Gram positive flora, and enrichment in Gram negative bacteria is the broadly emerging pattern in different types of IBD. *E. coli* is the predominant Gram negative species of the normal intestinal flora, where, as a commensal, it usually co-exists in good harmony with the host. Recognition of a high association of a more aggressive pathotype, Adherent Invasive *E. coli* with ileal CD strongly implies a role in etiopathogenesis of intestinal inflammation. The possibility of AIEC operating as a specific pathogen in IBD is also supported by their invasive behavior in Boxer dog Granulomatous Colitis.

We have yet to elucidate the specific mechanistic and temporal effects of dysbiosis in the development of IBD. The ranking order and interplay of the altered dominant floral groups with the other key etiologic factors in IBD, i.e. genetics, environment, and the immune system, are poorly understood. The critical questions that have yet to be addressed are whether dysbiosis is the primary initiating event, or occurs secondary to another trigger of acute intestinal inflammation. The potential for unresolved dysbiosis to result in chronic IBD is also unresolved. The research focus of this work has been aimed at advancing our understanding of host-bacterial interactions in the inflammatory bowel diseases. We hope that this in turn will impact disease management and uncover new strategies for therapeutic intervention.

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CHAPTER 2

Inflammation Drives Dysbiosis and Bacterial Invasion in Murine Models of Ileal Crohn's Disease

ABSTRACT

Elucidating the interplay between genetic susceptibility, the microbiome, the environment and the immune system in Crohn's disease (CD) is essential to optimize patient care. Here we sought to resolve these complex interrelationships in murine models of ileitis.

We demonstrate that severe inflammation induces a consistent shift from Gram positive to Gram negative bacteria and mucosal invasion by adherent and invasive *E. coli* (AIEC) that mirrors closely the dysbiosis of ileal CD. Superimposition of genetic susceptibility revealed greatest dysbiosis and bacterial invasion in the CD-susceptible genotype, $NOD2^{-/-}$, and reduced dysbiosis in ileitis-resistant $CCR2^{-/-}$ mice. Abrogating inflammation with the CD therapeutic anti-TNF-α mAb tempered dysbiosis and bacterial invasion.

We conclude that inflammation drives ileal dysbiosis and proliferation of AIEC. The identification of CCR2 as a target for therapeutic intervention, and discovery that host genotype and therapeutic blockade of inflammation impact the threshold and extent of ileal dysbiosis are of high relevance to CD.

INTRODUCTION

Inflammatory bowel diseases (IBD) encompass a complex of inflammatory intestinal disorders that are increasing in global prevalence and typified by recurrent flares of acute on chronic inflammation.[1-4] Traditionally, IBD comprises two distinct entities, Crohn's Disease (CD) and ulcerative colitis (UC),[1-6] both widely accepted to arise from the convergence of genetic susceptibility, enteric bacteria, the immune response, and environmental factors such as smoking, stress and diet.[6-13]

The best characterized genetic susceptibility locus in CD has been mapped to NOD2, and polymorphism in this gene occurs in 20-30% of CD patients.[14,15] The NOD2 gene encodes nucleotide-binding oligomerization domain 2, an intracellular pathogen recognition receptor (PRR) that, on sensing the bacterial cell wall antigen, muramyl dipeptide (MDP), evokes a pro-inflammatory response mediated by the NF-ƙB pathway.[16,17] The effect of NOD2 polymorphisms in CD are incompletely understood, but appear to culminate in impaired bactericidal defenses and unchecked intestinal inflammation.[18-20] More recently, genome-wide studies have shown CD-associated polymorphisms in autophagy-related 16-like protein 1 (ATG16L1) and immunity-related GTPase family, M, (IRGM), genes involved in killing intracellular microbes.[10, 21, 22] Clearly the common theme emerging in CD genetics involves the abnormal interfacing of innate immunity with intestinal microbes.[23]

Bacteria play a pivotal role in CD pathogenesis, purportedly via loss of immune tolerance to endogenous flora.[1,8,24,25] However more recent work shows global imbalances of the intestinal microbiome in CD, termed

‘dysbiosis,’ typified by a predominance of ‘aggressive’ species such as *Proteobacteria* relative to ‘protective’ species such as *Firmicutes.* A novel pathotype within the *Proteobacteria,* Adherent and Invasive *E. coli* (AIEC), is increasingly associated with CD in the US and Europe, and has been shown to invade and persist intracellularly within intestinal epithelial cells and macrophages.[26-29] A role for AIEC in CD is further supported by their ability to induce granulomas *in vitro* and to exploit host defects in bacterial elimination, as conferred by CD-associated polymorphisms in ATG16L, IRGM and NOD2.[30,31]

Here we examine the dynamics of the relationship between inflammation and the ileal microbiome. First, we evaluate different triggers for their capacity to induce ileal inflammation in C57BL6 mice: *Toxoplasma gondii*, *Giardia muris* and indomethacin. We show that moderate to severe ileitis is induced by *T. gondii* and indomethacin, and causes a consistent pattern of dysbiosis, characterized by reduced microbial diversity and a global shift in the ileal microbiome from >95% Gram positive, to >95% Gram negative species. Mucosal invasion by *E. coli* with an AIEC-pathotype accompanies severe ileitis and dysbiosis. Next we show that dysbiosis is significantly muted and bacterial invasion prevented when we limit the inflammatory response via deletion of a pro-inflammatory chemokine receptor, CCR2. In contrast, we observe heightened dysbiosis and *E. coli* invasion when we induce ileitis in the absence of NOD2. Lastly, abrogating inflammation with anti-TNF-α, a mainstay of CD management, limits the extent of dysbiosis and bacterial invasion. In summary, we establish that acute ileitis induces dysbiosis and proliferation of mucosally invasive *E. coli,* irrespective of trigger and host

genotype. We discover that therapeutic blockade of inflammation may indirectly control dysbiosis, and speculate that failure to completely resolve acute dysbiosis may set the scene for chronic microbial-driven inflammation in CD.

METHODS

Mice: Eight to 12 week old female C57BL/6 and Swiss Webster mice were purchased from Taconic Farms. Breeding pairs of CCR2$^{-/-}$ and NOD2$^{-/-}$ mice were purchased from The Jackson Laboratory. Mice were established under specific pathogen-free conditions in the Transgenic Mouse Facility at Cornell University, which is accredited by the American Association of Laboratory Animal Care.

Toxoplasma gondii infections: Type II low virulence *T. gondii* cysts (ME49) were obtained from chronically infected Swiss Webster mice by homogenizing brains in sterile PBS. Homogenate was passaged through an 18' needle, and cysts enumerated by phase contrast microscopy. Age-matched C57BL/6 mice were infected with 100 cysts by gavage, and ileum harvested at 4 (n=5, T4) and 8 (n=5, T8) days post-infection. Five uninfected control mice were gavaged with sterile PBS and ileum harvested on day 8.

Indomethacin: Indomethacin solubilized in PBS was administered by gavage to C57BL/6 mice at a low dosage (LDI) of 0.1mg/mouse/day (n=5) for 5 days, and a high dosage (HDI) of 1mg/mouse/day for 3 days (n=5). Ileum was harvested on day 7 (LDI) and day 3 (HDI).

Giardia muris infections: $2x10^5$ *G. muris* trophozoites were administered to C57BL/6 mice by gavage. Ileum was harvested at 7 (n=5, G7) and 14 days (n=5, G14) post-infection. Five uninfected control mice received sterile PBS by gavage and ileum was harvested on day 14.

Anti-TNF-α mAb treatment: Rat anti-mouse TNF-α (clone XT22.11) was purified from hybridoma supernatants by passage over a protein G sepharose column (Invitrogen). Mice were injected intraperitoneally with 3mg anti-TNF-α or 3mg of control rat IgG (Jackson ImmunoResearch) on days 3, 5 and 7 post-infection.

Histology: Hematoxylin and eosin stained sections of ileum were evaluated in a blinded manner by a pathologist. Severity of inflammation was graded according to cellular infiltrate, Paneth and goblet cell number, and architectural change.

Pyrosequencing: Pyrosequencing was based upon the bTEFAP method as described previously.[59-61] DNA was amplified using 27F-519R primers, labeled with linkers and tags, and pyrosequencing performed based upon manufacturer's instructions for Titanium chemistry FLX sequencing (Roche Applied Science, Indianapolis, IN). Data were curated so that only high quality sequence reads (Phred20) were used: sequences < 200bp were depleted, sequences with degenerate base calls were depleted, singletons were depleted, and the final data annotated with BLASTn.

Fluorescence in situ hybridization (FISH)**:** Formalin-fixed, paraffin-embedded sections of ileum mounted on Probe-On Plus slides (Fisher Scientific, Pittsburgh, PA, USA) were screened by eubacterial FISH with probes targeting

16S rDNA as previously described.[27,35] Subsequent hybridizations were informed by FISH and pyrosequencing data, employing EUB338 in combination with 1531-Cy3-5' or *E. coli* (Cy3-5', IDT, Coralville, IA, USA). Bacteria spotted slides were used to check probe specificity. Sections were examined with an Olympus BX51 epifluorescence microscope. Images were captured with a DP-70 camera and DP-Controller software and processed using DP Manager (Olympus America, Center Valley, PA, USA).

Quantitative PCR: Total bacteria and *E. coli* (uidA gene) were quantified as previously described.[27, 34] A standard curve was generated using DNA isolated from *E. coli* of known concentration, determined by quantitative plating. Bacterial DNA was normalized to biopsy size by quantification of murine 18S rRNA using the control kit RT-CKFT-18S (Eurogentec, Seraing, Belgium). 18S rRNA values were converted to cell number per kit instructions, and bacterial number was expressed as colony-forming unit (CFU)/10^6 murine cells.

E. coli characterization: *E. coli* strains were isolated from ileum as previously described.[27] For genotypic characterization, 3 to 5 *E. coli* colonies per biopsy were screened by random amplified polymorphic DNA PCR (RAPD-PCR) and the major phylogenetic groups (A, B1, B2 and D) determined by triplex PCR.[27] Isolates differing in overall genotype were selected for subsequent analyses and stored at -80°C. Fresh non-passaged bacteria were used for all investigations. Isolates were serotyped for OH antigens and screened at the *E. coli* Reference Center at Penn State University for heat-labile toxin, heat-stable toxins a and b, Shiga-like toxin types 1 and 2, cytotoxic necrotizing factors 1 and 2, and intimin-*γ*.[62] The presence of *ratA, pmt1, colV*, hemolysin co-regulated protein, long polar fimbriae, *lpfA154* and *lpfA141*, capsular

polysaccharide gene, *kpsMII*, yersinia bactin receptor gene, *fyuA*, gene *iss*, pathogenicity island associated maltose and glucose-specific IIABC component, *malX*, type II secretion protein, *gsp*, type IV secretion system protein, *traC*, afimbrial adhesion, *afaBC*, fimbrial proteins, *sfaDE*, *papC*, *focG*, and invasion gene *ibeA* were determined as previously described.[27, 63-68] The invasive abilities of *E. coli* isolates in Caco-2 cells, and intracellular survival and replication in J774 macrophages were determined as previously described.[27,35]

Statistical analysis: For comparisons involving >2 groups, Kruskal-Wallis and Mann-Whitney post-test were applied. Comparisons between 2 groups were performed using Mann-Whitney. Chi square was used to create the association plot for changes in relative proportions of bacterial families. Dendrograms were created in NCSS 2007. $P<0.05$ was considered significant.

RESULTS

T. gondii and indomethacin trigger acute ileitis

Granulomatous ileitis is present in around 70% of CD patients, thus our first aim was to establish clinically relevant murine models of ileitis using enteric infections (*T. gondii*, *G. muris*) and NSAID ingestion (indomethacin). *T. gondii* infection is known to induce ileitis with CD-like immunopathology in C57BL/6 mice, i.e. $CD4^+$ T-cell mediated inflammation dominated by Th1 cytokines TNF-α and IFN-γ.[32, 33] We show that peroral infection with *T. gondii* strain ME49 in C57BL/6 mice induces moderate to severe ileitis within 8 days of infection (T8, Figure 2.1a, 2.1b). Histologic changes comprised

granulomatous inflammation, villus atrophy, necrosis, and Paneth cell and goblet cell depletion, comparable to CD.[1, 32] In contrast, *G. muris* induced minimal to mild ileitis and villus hyperplasia within 14 days of infection (G14, Figure 2.1c). Peroral indomethacin induced dose-dependent injury; low dosage (LDI) caused mild ileitis, whereas high dosage (HDI, Figure 2.1d) caused death in 2 mice and moderate to severe ileitis in the 3 survivors. These results establish that we can induce ileitis of graded severity using environmental triggers of relevance to CD.

Acute ileitis is associated with dysbiosis and *E. coli* invasion.

To capture the microbial response to induced inflammation, we analyzed the composition and spatial distribution of the ileal microbiome by eubacterial 16S rDNA pyrosequencing and fluorescence in situ hybridization (FISH), targeting bacterial 16S rDNA. We discovered that a Gram positive (G+) bacterial population prevailed in control C57BL/6 mice, with 98% of sequences classed as phylum *Firmicutes* (Figure 2.2a, 2.2b, Tables 2.1 and 2.2). In contrast, moderate to severe ileitis in the *T. gondii* (T8) and indomethacin (HDI) models induced a dramatic shift ($p<0.0001$) to >99% G- bacteria, as shown in the association plot (Figure 2.2b). In T8, 92% of sequences were classed as *Proteobacteria* and 7% *Bacteroides.* In HDI, 75% of sequences were classed as *Proteobacteria* and 24% *Bacteroidetes.* Ileitis was also associated with marked loss of microbial diversity, from 25 genera in controls, to 11 and 13 genera in T8 and HDI respectively (Figure 2.3). On FISH analysis with eubacterial and *E. coli* probes we visualized large numbers of mucosa-associated bacteria and invasive *E. coli* in all T8 and HDI mice

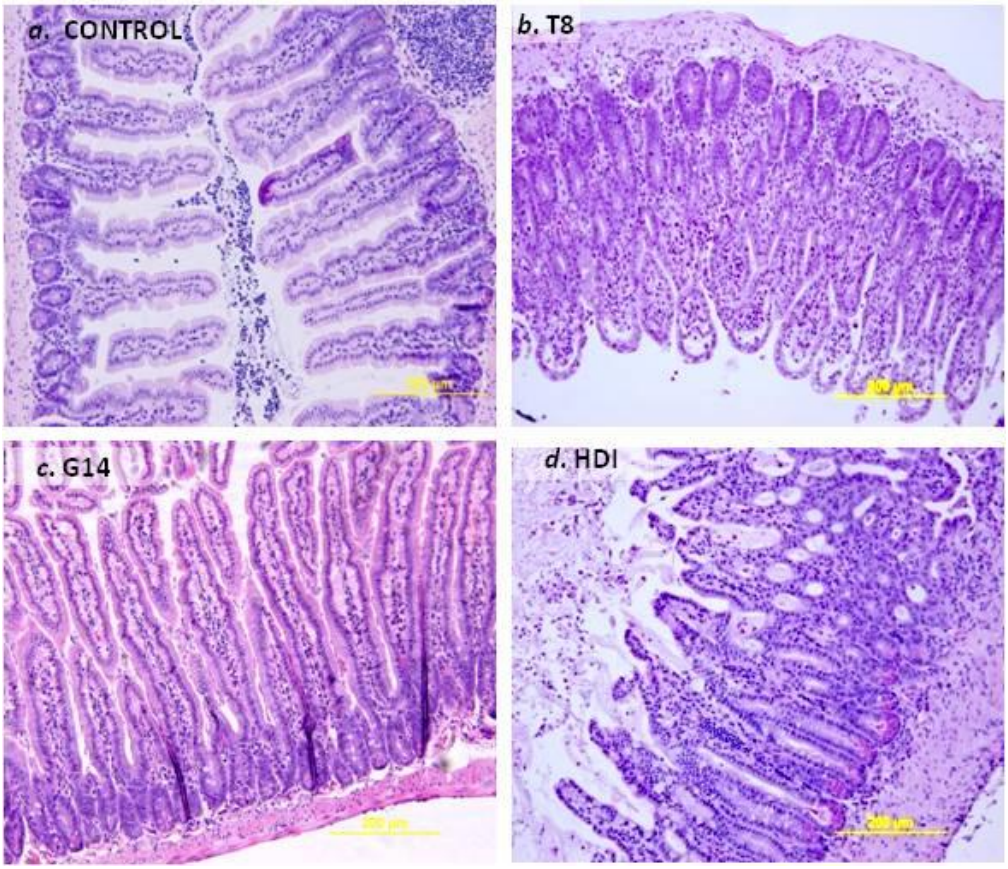
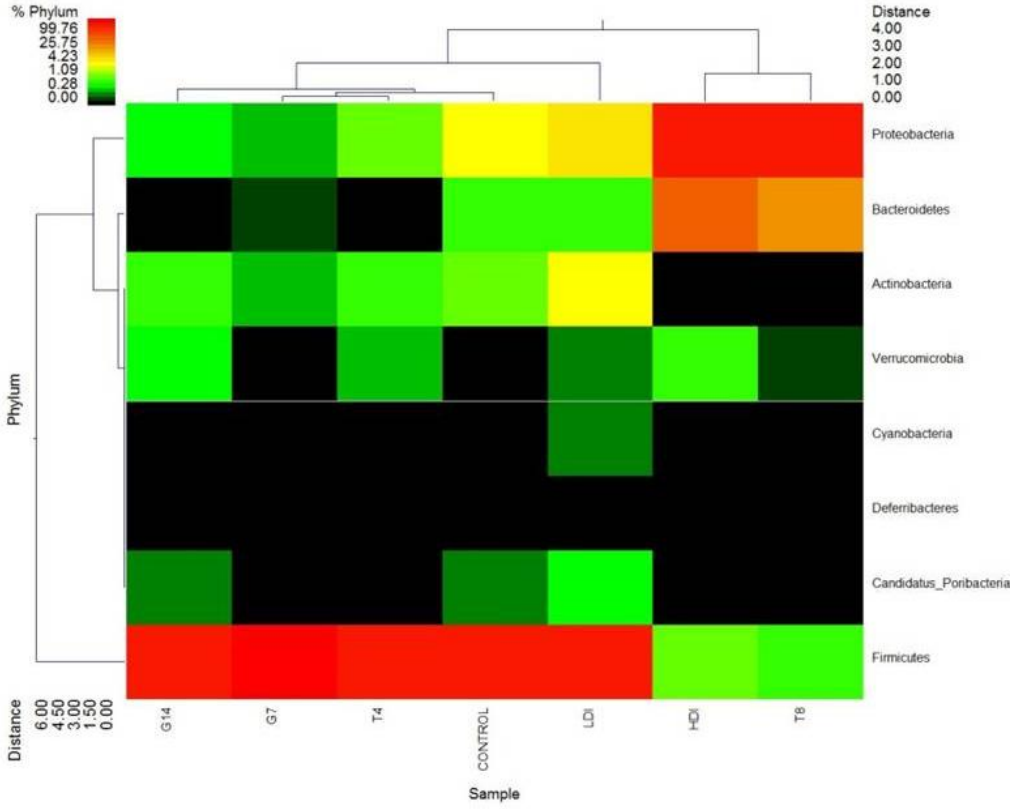


Figure 2.1: *T. gondii* infection (T8) and high dose indomethacin (HDI) trigger severe ileitis, dysbiosis and intramucosal *E. coli* invasion in C57BL/6 mice. **(a).** Normal ileal histology in control mice. **(b).** Moderate to severe ileitis develops in *T. gondii*-infected mice 8 days after peroral infection with 100 Type II ME49 cysts (T8) **(c).** *G. muris*-infected mice develop minimal inflammation and villus hyperplasia 14 days post-infection (G14). **(d).** High dose indomethacin (HDI, 1mg/mouse for 3 days by gavage) induces severe ileitis (H&E, 40x).

Figure 2.2: **(a)** Dendogram of 16S rDNA pyrosequencing reveals a Gram- shift associated with ileitis. Gram+ bacteria predominate in control mice and mice with mild inflammation (G7, G14: 7 and 14 days post-*Giardia* infection, T4: *T. gondii* 4 days post-infection; LDI: low-dose indomethacin). A floral shift to >95% Gram- bacteria dominated by the phyla *Proteobacteria* and *Bacteroidetes* ($p<0.0001$) is associated with moderate to severe ileitis in T8 and HDI mice. **(b)** Association plots of the number of sequences obtained by 16S rDNA pyrosequencing corresponding to bacterial families. Box size represents relative proportions of sequences. Relative to controls, red indicates sequence enrichment, whereas green indicates depletion, highlighting the ileitis-associated shifts from *Firmicutes (Lactobacillaceae, Clostridiales)* to *Enterobacteriaceae* (T8, HDI) and *Erysipelotrichaceae* (G7, G14, T4).

(a).


% Phylum
99.76
25.75
4.23
1.09
0.28
0.00
Distance
4.00
3.00
2.00
1.00
0.00
Proteobacteria
Bacteroidetes
Actinobacteria
Verrucomicrobia
Cyanobacteria
Deferribacteres
Candidatus_Poribacteria
Firmicutes
Phylum
Distance
6.00
4.50
3.00
1.50
0.00
G14
G7
T4
CONTROL
LDI
HDI
T8
Sample

(b)

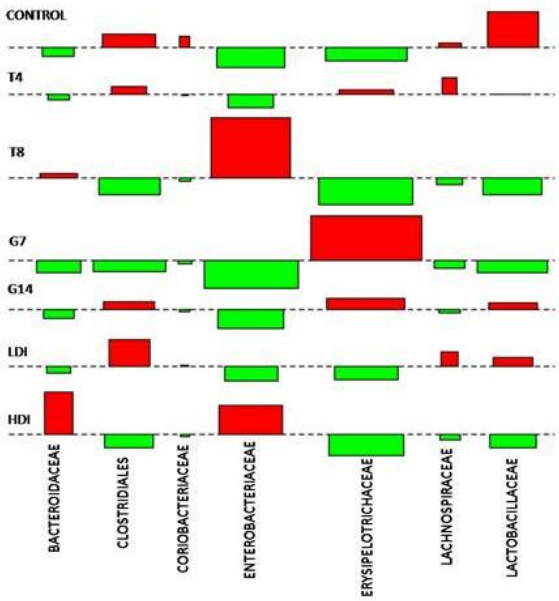

CONTROL
T4
T8
G7
G14
LDI
HDI
BACTEROIDACEAE
CLOSTRIDIALES
CORIOBACTERIACEAE
ENTEROBACTERIACEAE
ERYSIPELOTRICHACEAE
LACHNOSPIRACEAE
LACTOBACILLACEAE

Table 2.1: 16S rDNA pyrosequencing sequence classification (% of total sequence number, (n) by genus for ileitis trigger experiments (5 mice per group): Control - uninfected/untreated mice; T4, T8 - 4 and 8 days after T. gondii infection; G7, G14 - 7 and 14 days after G. muris infection. LDI, HDI: low dose (0.1mg/mouse) and high dose (1mg/mouse) indomethacin treatment.

	MOUSE GROUP						
GENUS	**CONTROL n=1898**	**T4 n=1970**	**T8 n=6101**	**G7 n=6102**	**G14 n=4099**	**LDI n=2930**	**HDI n=4903**
Clostridium	13.4	17.1	0.2	7.4	22.6	30.2	0.1
Turicibacter	8.9	44.9	0.0	86.2	52.3	8.8	0.0
Lactobacillus	53.4	13.7	0.0	5.7	21.0	23.2	0.1
Roseburia	7.5	8.8	0.0	0.2	1.1	4.4	0.0
C. Arthromitus	4.7	7.7	0.0	0.0	0.0	13.1	0.0
Papillibacter	2.7	0.3	0.0	0.0	0.5	3.3	0.0
Anaerostipes	0.0	0.0	0.0	0.0	0.0	0.8	0.0
Coprococcus	0.5	1.2	0.0	0.0	0.0	1.4	0.0
Ethanoligenens	2.0	1.7	0.0	0.0	0.7	1.2	0.0
Allobaculum	0.2	0.1	0.0	0.0	0.0	3.4	0.0
Anaerotruncus	0.8	0.0	0.0	0.0	0.0	0.7	0.0
Eubacterium	0.8	0.9	0.0	0.0	0.3	1.7	0.0
Citrobacter	0.1	0.2	1.0	0.0	0.0	0.6	1.6
Escherichia	0.2	0.0	17.6	0.0	0.0	0.6	48.6
Proteus	0.2	0.0	71.1	0.0	0.0	0.0	24.6
Bacteroides	0.2	0.0	7.0	0.0	0.0	0.2	23.3
Other	4.1	3.2	3.1	0.2	1.1	6.2	1.5

Table 2.2: 16S rDNA pyrosequencing data for all mouse groups, showing Shannon-Weaver bacterial diversity index, observed operative taxonomical units (OTU), the predicted maximum number of OTUs, rarefaction, and species richness estimators (ACE and Chao 1) at strain (1% dissimilarity), species (3%), and genus (5%) level.

Mouse group	# OF SEQUENCES	Rarefaction 1%	Rarefaction 3%	Rarefaction 5%	shannon 1%	shannon 3%	shannon 5%	otu 1%	otu 3%	otu 5%	ace 1%	ace 3%	ace 5%	chao1 1%	chao1 3%	chao1 5%
CONTROL	1898.0	145.9	81.6	69.1	3.5	2.6	2.1	150.0	83.0	70.0	168.8	66.9	50.4	166.3	62.6	48.4
T4	1970.0	162.1	83.0	70.6	3.3	2.0	1.9	170.0	86.0	73.0	173.1	71.7	61.6	203.2	72.0	58.8
T8	6101.0	161.0	48.7	28.8	3.0	1.8	1.0	162.0	49.0	29.0	218.0	50.5	25.4	216.1	46.0	23.0
G7	6102.0	156.0	61.5	44.7	3.1	1.0	0.7	157.0	62.0	45.0	219.7	83.3	62.6	204.0	72.4	53.4
G14	4099.0	178.5	94.1	69.4	3.3	1.7	1.5	180.0	95.0	70.0	217.4	97.0	79.0	200.4	96.0	68.0
LDI	2930.0	220.5	132.9	114.2	4.1	2.9	2.7	225.0	135.0	116.0	256.7	134.1	113.3	240.8	124.5	109.2
HDI	4903.0	169.3	49.6	31.8	3.5	2.4	1.8	171.0	50.0	32.0	218.7	57.9	36.7	228.3	61.8	35.8
CCR2$^{-/-}$0	3977.0	158.1	79.2	72.4	2.7	1.3	0.7	162.0	81.0	74.0	174.5	64.8	51.8	183.8	68.4	59.0
CCR2$^{-/-}$4	6102.0	137.1	43.7	36.7	2.6	0.7	0.3	138.0	44.0	37.0	201.1	73.7	66.3	210.1	63.0	56.1
CCR2$^{-/-}$8	1180.0	98.1	68.6	63.7	3.4	2.6	2.3	105.0	73.0	68.0	149.0	96.1	89.2	148.9	96.0	91.1
NOD2$^{-/-}$0	3579.0	169.5	78.3	69.3	3.1	1.8	1.6	171.0	79.0	70.0	223.9	98.6	88.6	205.0	95.3	84.6
NOD2$^{-/-}$4	1569.0	74.3	46.9	43.0	2.3	1.2	1.2	76.0	48.0	44.0	121.2	76.5	67.0	122.1	78.8	62.0
NOD2$^{-/-}$8	6102.0	163.3	24.9	7.9	3.7	2.3	0.4	164.0	25.0	8.0	199.1	27.8	12.1	193.9	31.0	11.0
Anti-TNF-α-0	4949.0	294.7	127.9	101.5	3.7	2.2	1.9	300.0	130.0	103.0	460.5	181.7	128.9	437.6	175.1	123.3
Anti-TNF-α-7	6101.0	147.2	38.7	25.9	3.0	1.7	0.6	148.0	39.0	26.0	173.5	40.0	22.3	170.0	36.2	20.5
IgG-7	3652.0	47.3	8.9	5.0	1.6	0.1	0.0	48.0	9.0	4.0	66.2	8.8	4.1	86.0	7.5	3.0

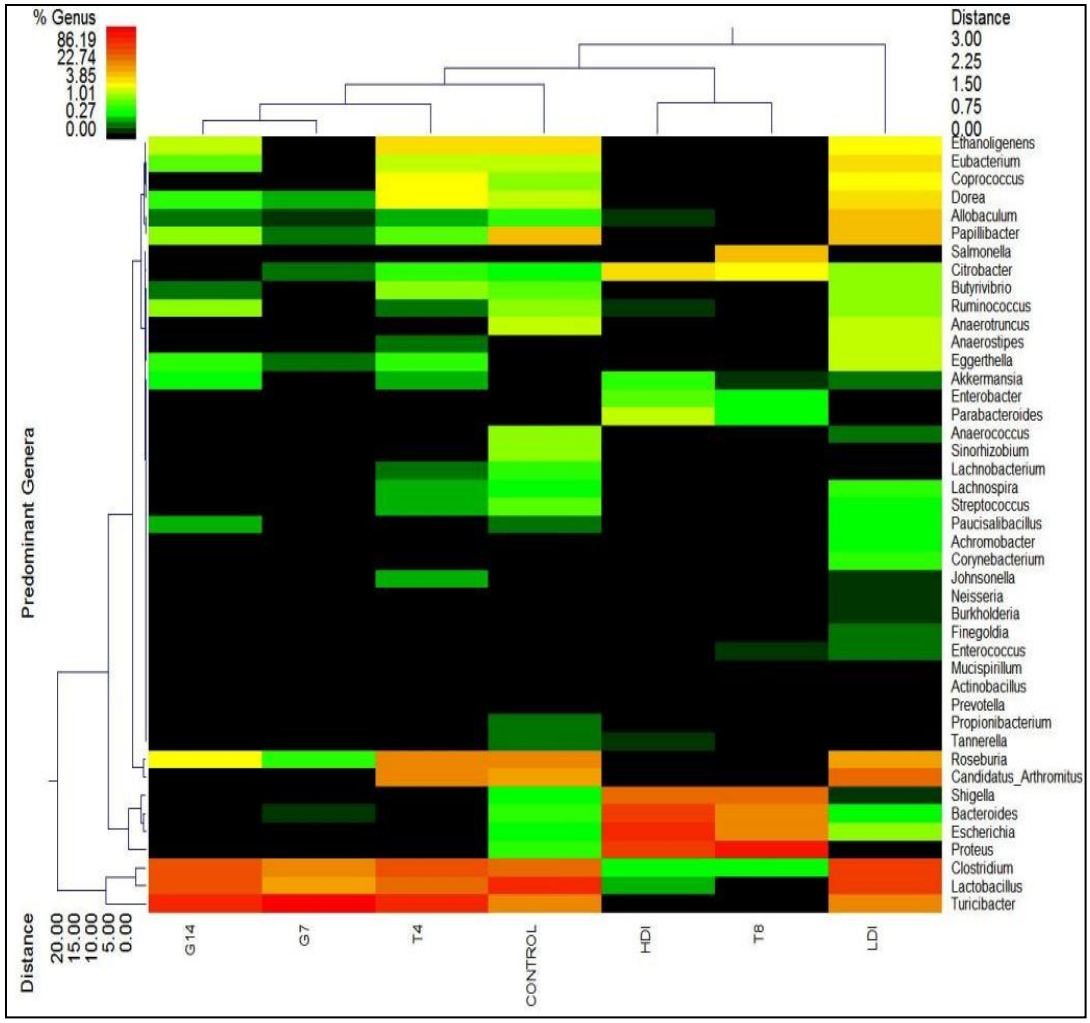


Figure 2.3: 16S rDNA sequencing by genera. Moderate to severe ileitis in T8 and HDI induces a Gram negative shift dominated by >99% *Proteobacteria* and loss of microbial diversity, from 25 genera in controls, to 11 and 13 genera in T8 and HDI respectively.

(Figure 2.4). In contrast, dysbiosis and bacterial invasion did not develop in mice with minimal (G7, G14) or only mild (T4, LDI) ileal pathology (Figures 2.2 and 2.3). We conclude that moderate to severe ileitis induces dysbiosis and *E. coli* invasion in the absence of CD-associated genetic susceptibility.

Severe ileitis is associated with *E. coli* proliferation.

We next quantified the numbers of total bacteria and *E. coli* in ileal tissue by real-time PCR, normalized to murine 18S genomic DNA.[27,34] In T8, the total bacterial load increased relative to controls ($p<0.01$), as did *E. coli* ($p<0.01$, Figure 2.5a, 2.5b). In addition, total bacterial load and *E. coli* number were significantly greater in T8 versus T4 (total bacteria, T8 vs T4 $p<0.01$, and *E. coli*, T8 vs T4 $p<0.01$). In HDI there was a significant increase in *E. coli* ($p<0.05$). In contrast, in mice with minimal to mild ileal pathology (G14, T4), the median total bacterial counts were actually lower than controls ($p<0.01$) and *E. coli* did not increase. In summary, inflammation modulates the number of total bacteria and *E. coli*.

Ileitis-associated *E. coli* strains are clonal and display an AIEC pathotype

E. coli was cultivable from 2/5 T4 mice with mild ileitis and all mice with moderate to severe ileitis, i.e. T8 (5/5), and HDI (3/3). All *E. coli* isolates from these different groups were phylogroup B1, and almost all (29/32) had an identical RAPD banding pattern (Figure 2.6 a). Representative strains from the dominant and minor RAPD groups were designated CUMT8 (serotype 08:H21) and CUMT4 (serotype 055:H8) respectively. CUMT4 was isolated rom a single mouse (T4) co-colonized with CUMT8 (Figure 2.6 a). Both CUMT8 and CUMT4 lacked the common virulence genes found in pathogenic

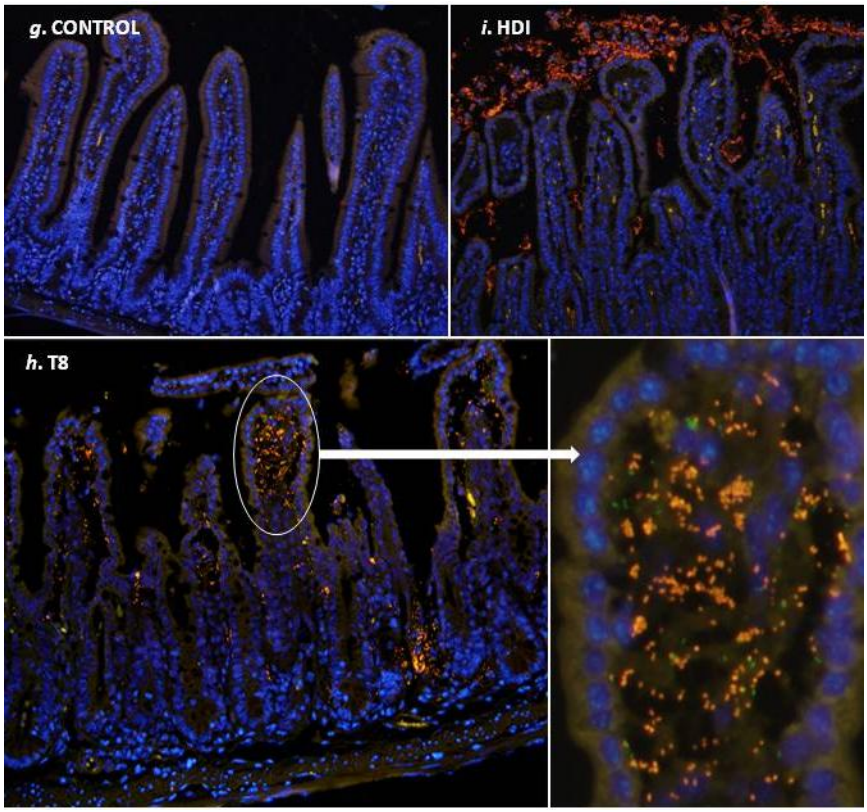
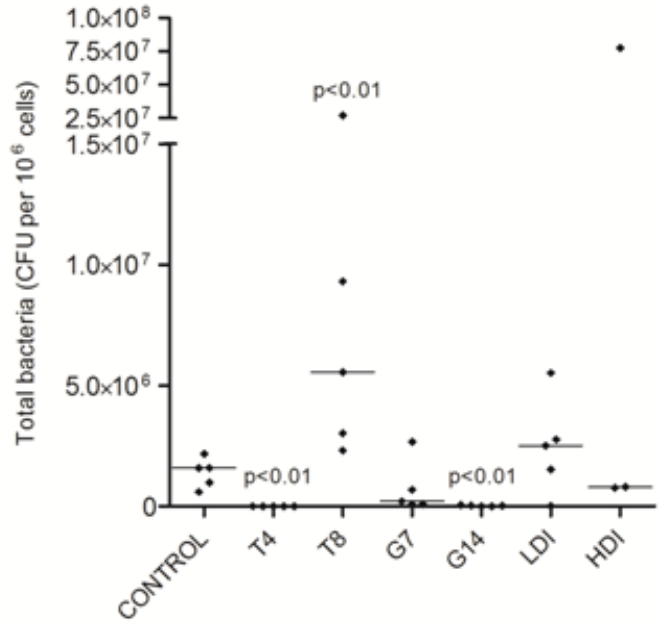


Figure 2.4: **(g)** FISH for eubacterial 16S rDNA reveals scant luminal and mucosa-associated flora in control mice (EUB338-Cy3, non-EUB338-6FAM with DAPI counterstaining, 40x). **(h, i)** Ileitis in T8 and HDI is associated with marked increases in the mucosal flora and invasive *E. coli* (*E*. *coli*-Cy3, EUB338-6FAM, 40x).

E. coli, and carried genes associated with AIEC (hemolysin co-regulated protein, long polar fimbriae, and a Type II secretion system).[27,35] Phenotypically, CUMT8 and CUMT4 behaved like the Crohn's-associated AIEC strain LF82 and were able to invade epithelial cells and persist within macrophages[29] (Figure 2.6b). These results demonstrate that ileitis of different etiologies induces proliferation of Adherent and Invasive *E. coli*.

(a).



(b).

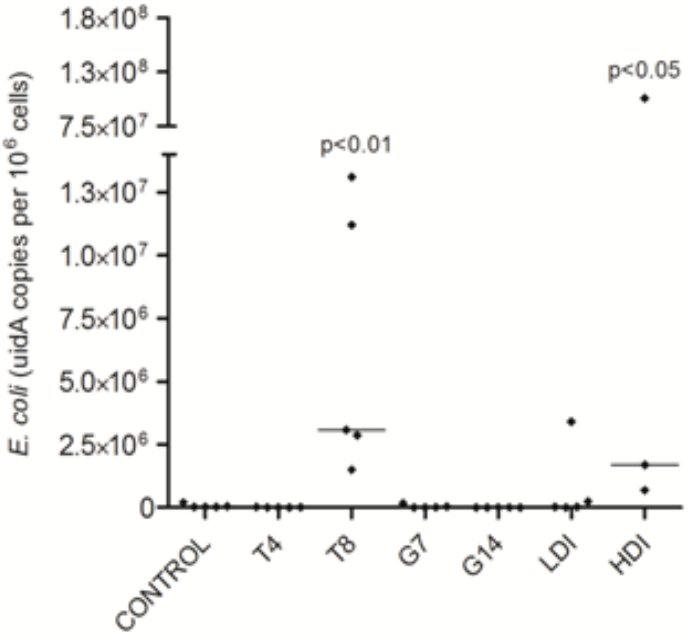
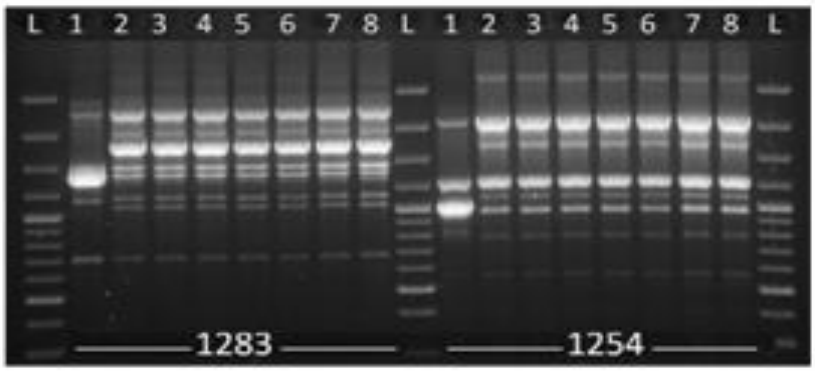


Figure 2.5: Ileitis-induced dysbiosis is associated with increased total bacterial number and clonal proliferation of adherent invasive *E. coli* (AIEC). **(a)** Total bacteria quantified by real-time PCR, expressed as bacterial CFU per 106 murine cells. Moderate to severe ileitis in T8 was associated with a significant increase in total bacteria relative to controls, whereas mild pathology (T4, G14) induced a significant decrease. **(b)** Moderate to severe ileitis (T8, HDI) induces *E. coli* proliferation (*E. coli* uidA quantification by real-time PCR).

(a).



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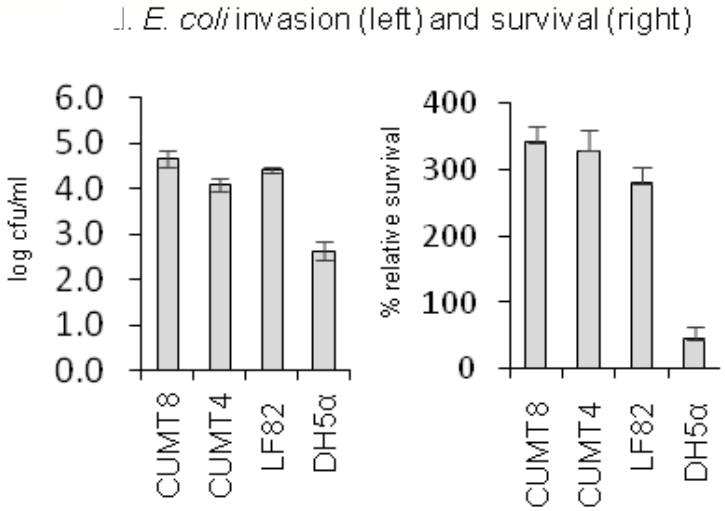

Figure 2.6: **(a)** Agarose gel electrophoresis showing the products of Random Amplification of Polymorphic DNA (RAPD-PCR) using primers 1283 and 1254. *E. coli* strains from: T4 (Lanes 1-3), T8 (4, 5) and HDI (6-8). Lane L, 100bp plus DNA ladder. *E. coli* isolates in lanes 2-8 are clonal, and a representative strain was designated CUMT8. *E. coli* in lane 1 was designated CUMT4. **(b).** Invasion and survival of *E. coli* CUMT8 and CUMT4 in cultured epithelial cells (Caco-2) and macrophages (J774). The ability of CUMT8 and CUMT4 to invade, and persist intracellularly was comparable to a CD-associated AIEC reference strain (LF82), and better than commensal *E. coli* DH5α.

Abrogating ileitis in CCR2$^{-/-}$ limits dysbiosis and prevents *E. coli* invasion

To further explore the interdependence of dysbiosis and inflammation, we evaluated the ileal microbiome in the setting of a limited host immune response, utilizing the *T. gondii* trigger model and CCR2$^{-/-}$ mice. Polymorphisms in CCR2 are associated with CD, and mice lacking the pro-inflammatory chemokine receptor CCR2 are protected from *T. gondii*-induced intestinal damage.[33] Here, ileitis in CCR2$^{-/-}$ at day 8 post-infection (CCR2$^{-/-}$8) was considerably less severe than T8 and HDI (Figure 2.7). 16S rDNA pyrosequencing in uninfected CCR2$^{-/-}$ (CCR2$^{-/-}$0) revealed 91% Gram positive *Firmicutes,* and 8% Gram negative *Bacteroidetes* (Figure 2.8, Tables 2.2 and 2.3). In CCR2$^{-/-}$8, 58% of sequences were Gram negative *Proteobacteria*, 27% *Bacteroidetes* and 14% *Firmicutes*. Despite this marked shift from 95% Gram positive flora in CCR2$^{-/-}$0, to 84% Gram negative flora in CCR2$^{-/-}$8 ($p<0.0001$), microbial diversity was actually increased in CCR2$^{-/-}$8, with 22 genera present at day 8 versus 18 genera at day 0 (Figure 2.9). Analysis by FISH in CCR2$^{-/-}$0 and CCR2$^{-/-}$8 revealed minimal mucosa-associated bacteria and no bacterial invasion (Figure 2.7b). Real-time PCR showed significantly increased *E. coli* at day 8 ($p<0.05$), but importantly this was much less than in T8 and HDI ($p<0.01$). A single *E. coli* strain was isolated from one CCR2$^{-/-}$8 mouse, and was clonal with CUMT8. We have shown that abrogating inflammation limits dysbiosis by maintaining microbial diversity, controlling bacterial proliferation, and preventing *E. coli* invasion.

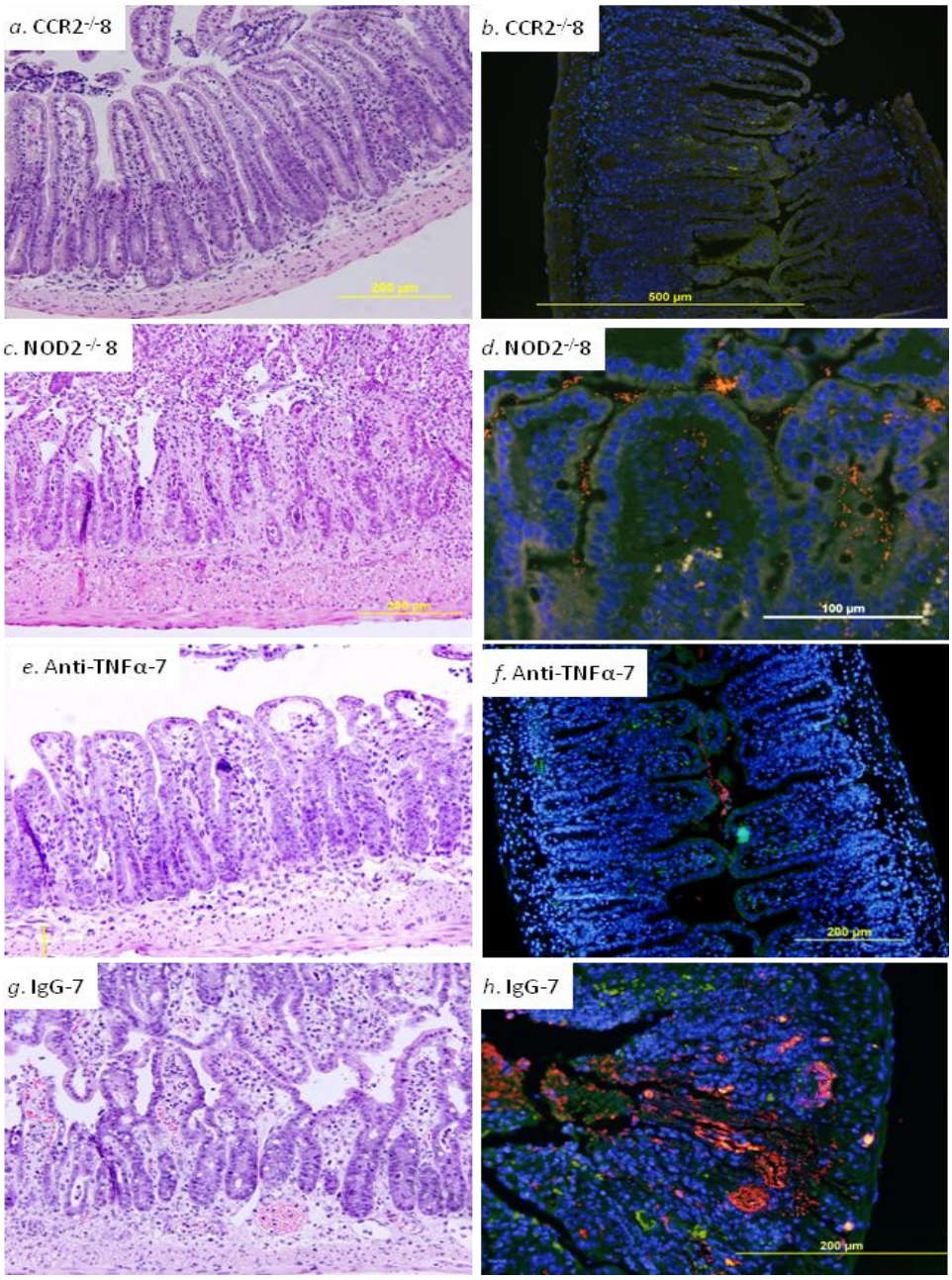

Figure 2.7: Dysbiosis and *E. coli* invasion are modulated by genetic susceptibility and pharmacotherapy. (a, b) CCR2$^{-/-}$8 are protected from *T.gondii*-induced ileitis and *E. coli* invasion. (c, d) NOD2$^{-/-}$8 develop severe ileitis and *E. coli* invasion. (e, f) anti-TNF-α mAb-7 reduces severity of ileitis and decreases bacterial invasion relative to IgG-7 control mice (g, h). a,c,e,g, H&E 40x; b, f - Cy3-EUB338/non-EUB338-6FAM; d, h, Cy3-*E.coli*/EUB338-6FAM, 40x.

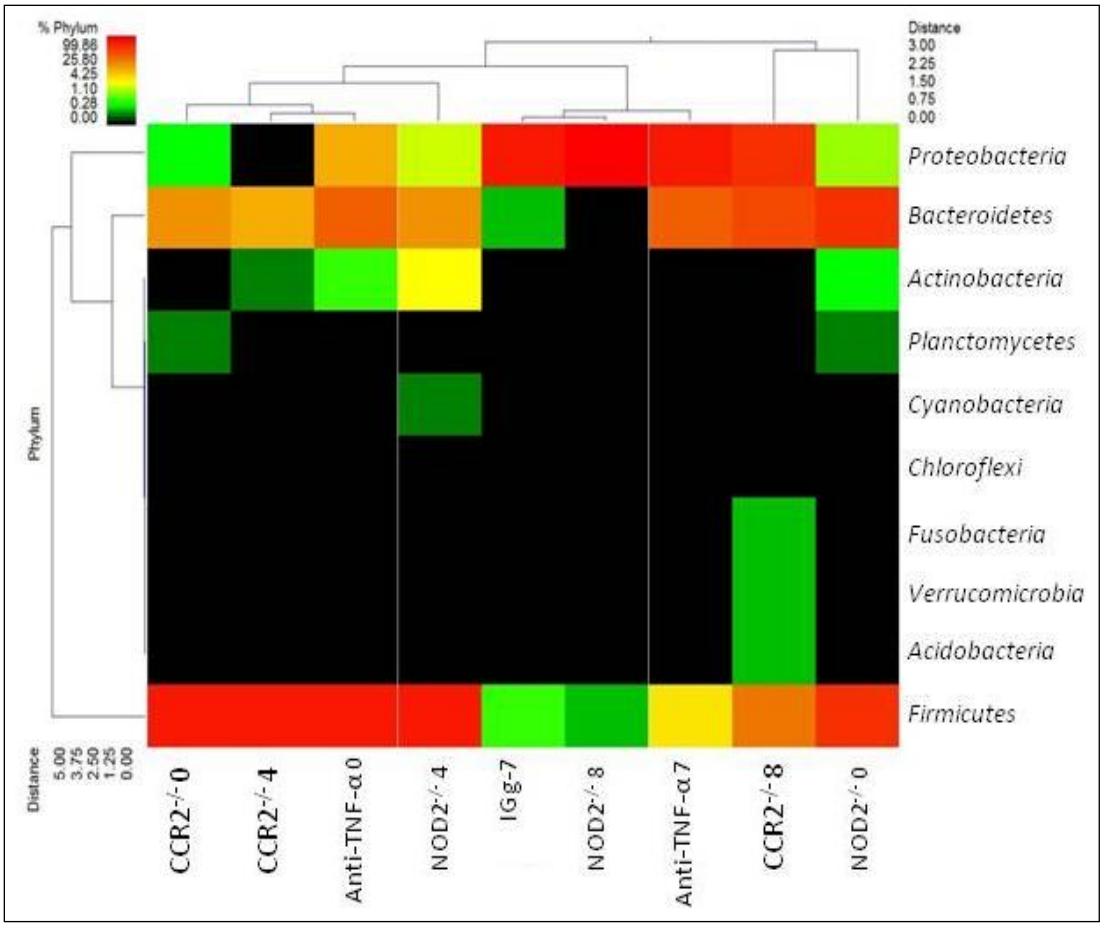

Figure 2.8: 16S rDNA pyrosequencing phylum clustering reveals that *T. gondii*-induced dysbiosis is modulated by genetic susceptibility and pharmacotherapy.

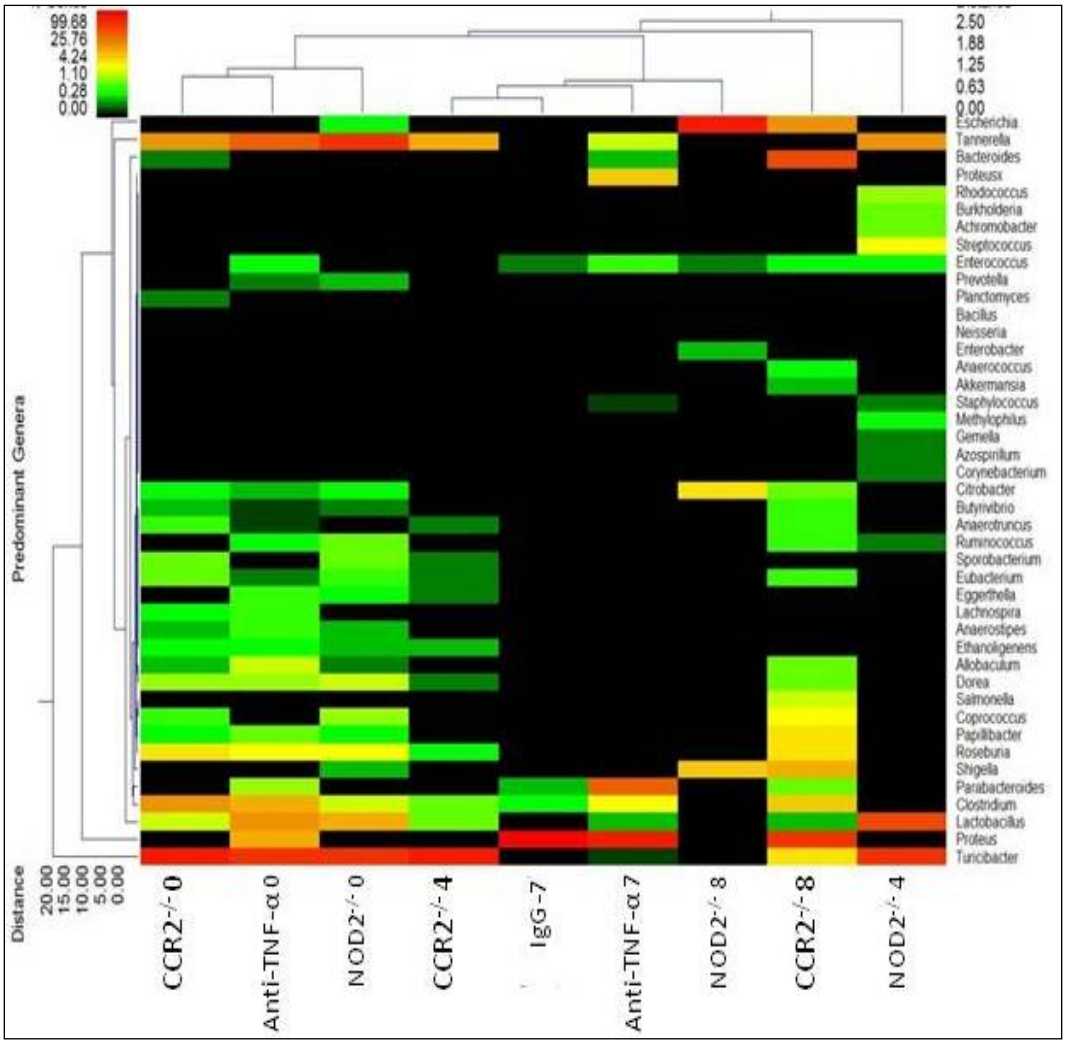

Figure 2.9: Abrogating ileitis by CCR2 deletion maintains microbial diversity ($CCR2^{-/-}$ 8), whereas enhancing ileitis by NOD2 deletion decreases diversity and increases *E. coli* proliferation ($NOD2^{-/-}$8). NOD2 deletion was also associated with a baseline shift to *Bacteroidetes* ($NOD2^{-/-}$0). (k) Anti-TNF-α mAb tempered dysbiosis (anti-TNF-α-7 versus IgG-7).

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Table 2.3: 16S rDNA pyrosequencing data (% of total sequence number, n) by genus for control, CCR2, NOD2 and anti-TNF-α mAb experiments (5 mice per group

	Mouse group											
GENUS	Control n=1898	T4 n=1970	T8 n=6101	$CCR2^{-/-}0$ n=3977	$CCR2^{-/-}4$ n=6102	$CCR2^{-/-}8$ n=1180	$NOD2^{-/-}0$ n=3579	$NOD2^{-/-}4$ n=3579	$NOD2^{-/-}8$ n-6102	Anti-TNF-α-0 n=4949	Anti-TNF-α-7 n=6101	IgG-7 n=3652
Clostridium	4.3	0.5	0.0	6.9	0.3	3.7	1.1	0.0	0.0	5.3	1.5	0.1
Papillibacter	3.4	0.0	0.0	0.1	0.0	2.6	0.1	0.0	0.0	0.4	0.0	0.0
Shigella	0.0	0.0	4.2	0.0	0.0	6.3	0.1	0.0	4.1	0.0	0.0	0.0
Escherichia	0.0	0.0	14.4	0.0	0.0	8.7	0.2	0.0	93.3	0.0	0.0	0.0
Dorea	2.3	0.2	0.0	0.6	0.1	0.3	0.7	0.0	0.0	0.7	0.0	0.0
Streptococcus	0.0	1.1	0.0	0.0	0.0	0.0	0.0	1.3	0.0	0.0	0.0	0.0
Tannerella	0.1	0.0	0.0	7.8	5.1	0.0	46.0	7.0	0.0	21.3	1.1	0.0
Turicibacter	23.0	71.2	0.0	79.4	93.3	2.5	42.2	60.1	0.0	52.0	0.0	0.0
Citrobacter	0.2	0.0	0.0	0.1	0.0	0.4	0.2	0.0	2.5	0.1	0.0	0.0
Proteus	0.0	0.1	75.3	0.0	0.0	41.5	0.0	0.0	0.0	5.8	76.1	99.7
Anaerotruncus	1.4	0.0	0.0	0.2	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0
Roseburia	3.8	0.1	0.0	2.3	0.1	1.8	1.2	0.0	0.0	1.1	0.0	0.0
Parabacteroides	0.3	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.5	19.8	0.1
Lactobacillus	20.9	18.4	0.0	0.7	0.3	0.1	5.4	28.4	0.0	10.1	0.1	0.0
C. Arthromitus	32.5	4.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Bacteroides	1.0	0.0	2.6	0.0	0.0	26.3	0.0	0.0	0.0	0.0	0.1	0.0
Other	6.8	4.1	3.5	1.7	0.6	5.1	2.9	3.3	0.2	2.7	1.3	0.1

The CD-susceptible genotype $NOD2^{-/-}$ lowers the threshold for dysbiosis.

To evaluate the impact of a CD-susceptible genotype on the inflammatory microbiome, we applied the *T. gondii* ileitis model to $NOD2^{-/-}$ mice. This resulted in the most severe inflammation relative to all other groups ($NOD2^{-/-}$8, Figure 2.7c). 16SrDNA pyrosequencing of uninfected mice showed a shift in the endogenous flora, with 53% *Firmicutes* and 46% *Bacteroidetes* in $NOD2^{-/-}$0, relative to 98% *Firmicutes* in uninfected wildtype mice (Figures 2.8 and 2.9, Tables 2.2 and 2.3). By day 8 post-infection we observed a dramatic Gram negative shift to 99.8% *Proteobacteria*. Microbial diversity also decreased the most, from 19 ($NOD2^{-/-}$0), to only 6 genera ($NOD2^{-/-}$8, Figure 2.9). Large numbers of mucosa-associated bacteria and invasive *E. coli* were observed in all $NOD2^{-/-}$8 mice (Figure 2.7d), and *E. coli* increased from $1.13x10^3$ uidA copies/10^6 cells in $NOD2^{-/-}$0, to $1.19x10^4$ in $NOD2^{-/-}$8. These results reveal that $NOD2^{-/-}$ perturbs the endogenous flora and enhances inflammation and dysbiosis in response to an injurious trigger.

Anti-TNF-α mAb limits dysbiosis and reduces bacterial invasion

Biological agents targeting the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α), are increasingly used in the management of CD.[36] Thus, our final aim was to explore the impact of anti-TNF-α mAb on the inflammation-dysbiosis dynamic. Mice treated with anti-TNF-α mAb developed less severe inflammation 7 days after *T. gondii* infection (anti-TNF-α-7) than controls which received treatment with an irrelevant antibody, immunoglobulin G (IgG-7, Figure 2.7).

16S rDNA pyrosequencing revealed a marked floral shift and loss of microbial diversity, from 71% *Firmicutes*, 21% *Bacteroidetes*,

6% *Proteobacteria* and 21 genera in uninfected controls (anti-TNF-α-0), to 99.7 % *Proteobacteria* (99% *Proteus* spp*.),* and 4 genera in *T. gondii*-infected mice administered IgG (IgG-7, Figure 2.8, 2.9, Tables 2.2 and 2.3). In anti-TNF-α-7, dysbiosis and loss of microbial diversity were tempered: 72% *Proteobacteria* (72% *Proteus spp.*), 21% *Bacteroidetes*, and 10 genera. We observed far greater mucosa-associated and invasive bacteria in IgG-7 (5/5 invasive) relative to anti-TNF-α-0 (0/5 invasive), and anti-TNF-α-7 (low numbers of intramucosal bacteria in 3/5). Interestingly, the vast majority of the invading bacteria in IgG-7 and anti-TNF-α-7 did not hybridize to an *E. coli* probe. Based on pyrosequencing results and the overgrowth of *Proteus* spp. in bacterial cultures, we suspect these invasive bacteria are a different member of the *Proteobacteria*, *Proteus* spp. This contrasts with invasive *E. coli* in T8 and HDI, and likely reflects the relative abundance of *Proteus* in the endogenous flora of mice in this experiment. These results show that anti-TNF-α treatment lessens the severity of inflammation, and while not completely protective, decreases the inflammation-induced floral shift by maintaining greater microbial diversity and reducing bacterial invasion.

DISCUSSION

The evidence is compelling that CD pathogenesis involves interplay between the intestinal microbiome, genetic susceptibility (e.g. NOD2), the immune system, and environmental risk factors. Our limited understanding of these interrelationships has yet to reconcile the wide spectrum of CD phenotypes, but mapping their interactions is pivotal to understanding CD pathogenesis and identifying new therapeutic targets. Here we establish that

acute ileitis induces a consistent shift in the enteric microflora from *Firmicutes* to *Proteobacteria*, accompanied by a reduction in microbial diversity and proliferation of AIEC. This 'inflammatory microbiome' recapitulates the dysbiosis of ileal CD.[24,27,37-40] When we superimposed a CD-susceptible genotype, NOD2$^{-/-}$, on *T. gondii*-induced ileitis, we enhanced inflammation and dysbiosis. Conversely, dysbiosis was tempered when we limited inflammation by CCR2 gene deletion, revealing a new potential therapeutic target in CD. Using anti-TNF-α mAb, a mainstay of CD therapy, we discover that dysbiosis can be reduced by pharmacologic manipulation of mucosal inflammation. We speculate that failure to resolve the inflammatory microbiome after acute non-specific enteric injury may stimulate persistent microbial-drive inflammation in CD.

Many IBD susceptibility genes have been discovered, but similar advances in defining environmental risk factors have lagged.[12] Animal models of CD are usually chemically-induced, genetically engineered, or congenic rodent strains with limited relevance to the clinical setting and disease phenotype.[6,41] To specifically examine the interrelationship between the microbiome and inflammation in ileal CD, which affects 70% of patients, we utilized murine models of ileitis incorporating environmental triggers of relevance to CD. NSAID ingestion, infectious enteritis and stress are known IBD risks.[42,43] While *T. gondii* is not typically associated with CD, it induces granulomatous ileitis of Th1-type immunopathology in C57BL/6 mice that mimics ileal CD.[32,44] The development of inflammation in this model is also microbial dependent.[5,44] Using these diverse stimuli we showed that the inflammatory microbiome is a common endpoint of ileitis, driven by severity of

inflammation rather than the trigger. Our finding that mild ileitis (T4, G14) was associated with decreased bacterial load suggests an ability to counteract dysbiosis until inflammation overwhelms. The villus hyperplasia observed in G14 mice raises the possibility that up-regulation of anti-microbial mucosal defenses may occur with mild injury.[45]

The question that naturally arises is whether the floral shift *per se* is in fact harmful, or a benign consequence of inflammation. However, consider that we show the degree of dysbiosis to directly relate to the severity of ileitis, and that invasive *E. coli* is always, and only, present in mice that develop a >95% Gram negative shift. Further, the selection pressure exerted by inflammation is sufficiently strong to drive clonal proliferation of a single AIEC strain (CUMT8) in all mice with severe ileitis, independent of stimulus. Here we show for the first time that acute ileitis induces the proliferation of mucosally invasive *E. coli* with an AIEC pathotype.[27,29,35] Taken together, these findings imply that dysbiosis exerts a damaging effect rather than occurring as an innocent bystander.

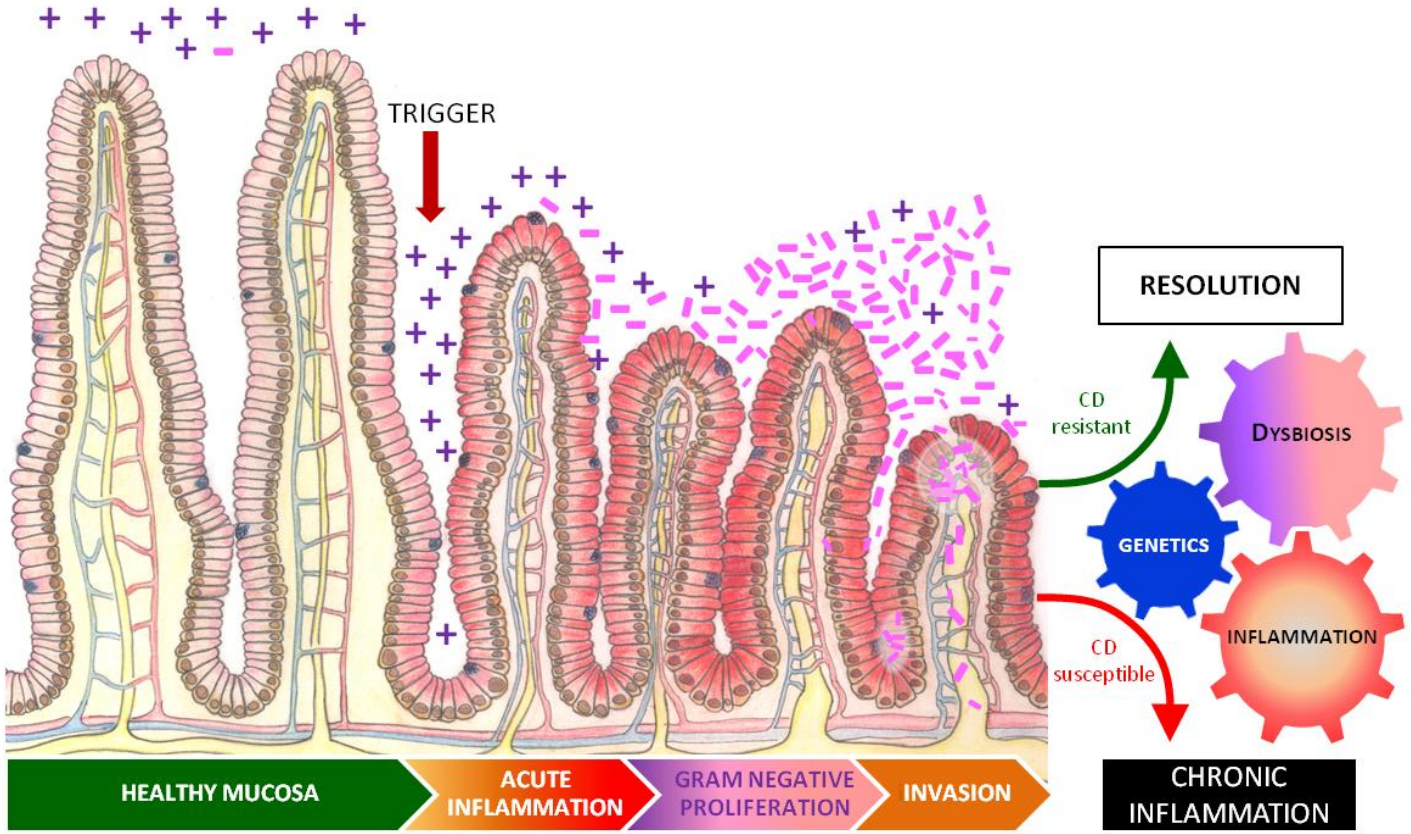
Increasingly implicated in CD in the US and Europe, AIEC have been isolated from the ileum of 36-38% of CD patients versus 6% of controls, and are associated with the severity of ileal CD.[27,46] Surprisingly little is known about the luminal microenvironment that promotes the proliferation of aggressive species such as *E. coli* and disappearance of protective *Firmicutes* such as *Fecalibacterium prauzsnitzii*. A plethora of inflammation-associated factors could favor, or fail to regulate bacterial proliferation and virulence.[46,47] Specific mechanisms implicated in the adhesion and invasion of AIEC include bacterial factors such as flagellin[48] and Type I pili,[49] as well as host factors

such as cell adhesion molecule CEACAM6[50,51] and the stress response protein Gp96.[51] Up-regulation of CEACAM6 and interaction with type 1 pili to promote adhesion and invasion is a potential pathomechanism for AIEC, but cannot account for our findings since mice do not express CEACAM6.[50] Notably, the highest *E. coli* counts were observed in mice with the most severe inflammation and dysbiosis, suggesting that quantification of mucosal *E.coli* may be a useful adjunct for monitoring CD activity (Figure 2.10).

When we superimposed the effect of host genotype on the inflammation-dysbiosis dynamic, we saw the most severe ileitis, dysbiosis, and *E. coli* invasion, in NOD2$^{-/-}$ mice. We postulate that this is attributable to altered sensing of bacterial MDP and reduced killing of luminal and intracellular *E. coli*.[14] Our observation of more *Bacteroidetes* sequences in uninfected NOD2$^{-/-}$ than all other control groups suggests a pre-existing baseline of floral shift.[52] Our findings indicate that NOD2 mutation confers greater vulnerability to inflammation and dysbiosis but importantly, absence of NOD2 was not a prerequisite for dysbiosis and invasion. This echoes the scenario in CD, where 60%–70% of CD patients show no NOD2 mutations.[14,53] Moreover, studies of identical twins, that basically eliminate the variable of host genetics, elegantly show that microbial composition is determined by presence or absence of CD rather than genetic make-up.[40,54]

The interdependence of inflammation and dysbiosis is further supported by our findings in inflammation resistant CCR2$^{-/-}$ mice. CCR2 is a chemokine receptor for monocyte chemoattractant proteins (MCP1-4) and we have shown previously that mice lacking CCR2 are protected from *T. gondii*-induced ileal pathology.[33] The CCR2$^{-/-}$ model is highly relevant to CD pathogenesis

Figure 2.10: Inflammation drives dysbiosis, Gram negative proliferation and *E. coli* invasion. Independent of genotype, ileitis induces a progressive decrease in microbial diversity, a shift from *Firmicutes* (**+**) to *Proteobacteria* (**-**), and proliferation of AIEC. Superimposition of genetic susceptibility on the inflammation-dysbiosis dynamic can lower (i.e. $NOD2^{-/-}$ genotype) or increase (i.e. $CCR2^{-/-}$ genotype) the threshold for dysbiosis in response to an external trigger. We speculate that genetic susceptibility may also influence the ability of an individual to resolve the self-perpetuating cycle of dysbiosis and inflammation generated by an acute insult.


TRIGGER
RESOLUTION
CD
resistant
DYSBIOSIS
GENETICS
INFLAMMATION
CD
susceptible
HEALTHY MUCOSA
ACUTE
INFLAMMATION
GRAM NEGATIVE
PROLIFERATION
INVASION
CHRONIC
INFLAMMATION

because CCR2+ lamina propria lymphocytes are a specific feature of ileal CD[55] and polymorphisms in CCR2 have been associated with CD in several studies.[56,57] On this basis we suggest that pharmacologic blockade of this receptor, which has been demonstrated in mice,[58] may provide a novel pathway for therapeutic intervention in CD. In a similar vein, by controlling inflammation using anti-TNF-α mAb, we reduced dysbiosis, unmasking an effect of this therapy not previously appreciated.

In summary, this study has explored the dynamics of the relationship between ileal inflammation and alterations in the intestinal microbiome termed dysbiosis. On the basis of our observations we propose a model where the composition and spatial distribution of the ileal microbiome is regulated by inflammation independent of the injurious trigger (Figure 2.10). Inflammation drives a progressive decrease in microbial diversity, a shift from Gram + to Gram -, and the proliferation of mucosally invasive bacteria such as adherent and invasive *E. coli.* While the common end-point of inflammation is the inflammatory microbiome, genetic susceptibility can impact the threshold for dysbiosis in response to an external trigger. We speculate that genetic susceptibility may also influence the ability of an individual to resolve the self-perpetuating cycle of dysbiosis and inflammation generated by an acute insult.

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CHAPTER 3

Antimicrobial Resistance Impacts Clinical Outcome

of Granulomatous Colitis in Boxer Dogs

ABSTRACT

E. coli have recently been identified within the colonic mucosa of Boxer dogs with granulomatous colitis (GC). Eradication of invasive *E. coli* is associated with clinical and histological remission. To optimize therapy against GC-associated *E. coli* we determined the antimicrobial susceptibility profiles of *E. coli* strains from GC and healthy dogs, and the effect of antimicrobial resistance on clinical outcome. *E. coli* was cultured from GC biopsies of 14 dogs and rectal mucosal swabs of 17 healthy dogs. Individual strains were selected by phylogroup and overall genotype, determined by triplex- and RAPD PCR respectively. Antimicrobial resistance was determined by broth microdilution MIC.

Culture yielded 23 *E. coli* strains from GC (1-3/dog, median 2) and 34 strains from healthy (1-3/dog, median 2). *E. coli* phylogroups were similar (p=0.18) in GC (5A, 7B1, 5B2, 6D) and healthy (2A, 10B1, 15B2, 7D). Resistance to ampicillin, amoxicillin-clavulanate, cefoxitin, tetracycline, trimethoprim-sulfa (TMS), ciprofloxacin and chloramphenicol was more common ($P<0.05$) in GC than healthy. Enrofloxacin resistant *E. coli* were isolated from 6/14 GC vs. 0/17 healthy ($P<0.001$). Of the enrofloxacin resistant cases, 4/6 were also resistant to macrophage-penetrating antimicrobials such as chloramphenicol, rifampicin, and TMS. Enrofloxacin treatment before

definitive diagnosis was associated with antimicrobial resistance and poor clinical outcome ($p<0.01$).

Antimicrobial resistance is common among GC-associated *E. coli* and impacts clinical response. Antimicrobial therapy should be guided by mucosal culture and antimicrobial susceptibility testing rather than empirical wisdom.

INTRODUCTION

Granulomatous colitis (GC), also known as histiocytic ulcerative colitis (HUC), is typically seen in young Boxer dogs < 4yrs of age, and was first described by Van Kruiningen in 1965.[1] Affected dogs usually present with signs of colitis (hematochezia, diarrhea, mucus, tenesmus) that is often accompanied by ill-thrift, weight loss, anemia and hypoalbuminemia. Diagnosis is based on colon histology, and typified by large numbers of periodic acid-Schiff (PAS)-positive macrophages, lymphocytes, plasma cells, eosinophils, epithelial ulceration and loss of goblet cells.[1, 2]

Recent studies have documented dramatic clinical responses to enrofloxacin, [3-6] and an association between GC and intramucosal *E. coli* in affected Boxer dogs.[5,7] Bacterial localization using fluorescence in situ hybridization (FISH) with an *E. coli* probe typically reveals multifocal clusters of invasive bacteria within the mucosa and intracellularly within macrophages.[5,7] Given the susceptibility of the Boxer breed, it appears likely that an underlying host defect in mucosal immunity enables opportunistic invasion by resident *E. coli.* [4] A role for the resident enteric microflora, particularly the

Enterobacteriaceae, is increasingly recognized in the pathogenesis of inflammatory bowel diseases (IBD), and increased numbers of mucosa-associated *E. coli* and serological responses against *E. coli* are observed in people with Crohn's disease (CD).[7-14] Remarkably, the *E. coli* strains isolated from Boxer dogs display a similar pathotype in cultured intestinal epithelial cells and macrophages to a novel group of *E. coli* strains, termed **A**dherent and **I**nvasive ***E. c****oli* (AIEC), that are associated with ileal inflammation in CD patients. [4, 7-9,15]

Traditionally, empirical treatment of GC with a variety of antimicrobials and immunosuppressive agents was met with poor success. We suspect that the positive clinical responses[3-6,16] reported in GC affected Boxer dogs treated with enrofloxacin or chloramphenicol reflects the susceptibility of *E. coli* to these antimicrobials, and their ability to accumulate in *E. coli* colonized macrophages.[17] This is supported by the correlation between clinical response to enrofloxacin and eradication of intramucosal and intracellular *E. coli.*[4, 5] To optimize antimicrobial selection against GC-associated *E. coli* we sought to determine the antimicrobial susceptibility profiles of *E. coli* strains isolated from GC and healthy dogs, and the effect of antimicrobial resistance on clinical outcome.

METHODS

Animals and sampling

Formalin fixed, paraffin embedded colonic mucosal biopsies from 14 Boxer dogs (8 M, 6 F; median age 12 mo., range 4-60 mo., ‘GC’ group) with

histologically confirmed GC were evaluated for intracellular *E. coli* by FISH analysis using eubacterial (EUB338-6FAM) and *E. coli*-specific probes (*E. coli*-Cy3), targeting 16S ribosomal DNA as previously described.[4] Biopsy samples for microbial culture were collected into Luria-Bertani (LB) broth with 20% glycerol on ice and stored at –80°C until processing. Clinical and outcome data were obtained from referring veterinarians and owners where possible. Affected dogs were classified as 'complete responders' if their clinical signs had completely resolved with antimicrobial treatment; 'partial responders' if their clinical signs had reduced in frequency or severity, and 'non-responders' if treatment had failed to elicit any improvement in clinical signs. Rectal mucosal swabs from 17 clinically healthy pet dogs (group 'H') of various breeds (6M, 11F; median age 60 mo, range 6-132 mo.) were inserted into BBL™ Port-a-cul™ transporter tubes (BD Diagnostics, MD) and processed immediately. The rationale for sampling *E. coli* was principally based on consideration of the localization of *E. coli* in biopsies evaluated by previous studies.[5,7] In healthy dogs *E. coli* are not invasive, and are restricted to superficial mucus and mucus containing glands, in contrast to dogs with GC where the invasive *E. coli* are located intramucosally and within macrophages. Hence we considered that collection of *E. coli* by biopsy in GC and swab in H would provide a representative sampling of mucosa-associated strains in each population. A secondary consideration that impacted our choice of methodology was the lack of justification for performing endoscopic biopsy on healthy client-owned dogs.

Isolation and characterization of *E.coli*

For microbial culture, biopsies were aseptically transferred from collection vials into a disposable tissue grinder and homogenized. The tissue homogenate or swabs were used to inoculate trypticase soy agar with 5% sheep blood and Gram negative broth (GN). Media were incubated at 37°C for 18-24 hours in 6% CO_2 and visually inspected for bacteria before being subcultured onto Levine EMB agar and MacConkey MUG agar containing the chromogenic indicator methylumbelliferyl-β-D-glucuronidase, and incubated at 37°C for 18-24 hours. Aerobic bacterial colonies were screened by Gram stain, catalase, and oxidase reaction, and were identified using the computer controlled, automatic, Sensititre System (TREK Diagnostic Systems). Conventional biochemical reactions using standard identification strategies were used as needed to supplement the Sensititre identification system, and confirmed by PCR targeting the *E. coli* housekeeping gene uidA (beta-glucuronidase).[9] Ten to 15 individual *E. coli* colonies per sample were selected at random and screened by triplex PCR for major *E. coli* phylogenetic groups (A, B1, B2, and D),[18,19] and genotyped using Random Amplified Polymorphic DNA (RAPD)-PCRs with primer 1283, followed by additional primers (1254 and 1290) on strains with a similar banding pattern. *E. coli* strains that differed in genotype were archived at –80°C, and fresh non-passaged bacteria were used for subsequent investigations.[4]

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MIC) of *E. coli* to selected antimicrobials was determined by the microdilution method and interpreted according to Clinical Laboratory Standards Institute (CLSI) interpretive criteria (CLSI, 2004; formerly NCCLS). The panel of antimicrobials tested included amikacin, amoxicillin-clavulanic acid (amox-clav), ampicillin, cefazolin, cefoxitin, cefpodoxime, chloramphenicol, ciprofloxacin, clindamycin, enrofloxacin, erythromycin, gentamicin, imipenem, marbofloxacin, orbifloxacin, streptomycin, tetracycline, and trimethoprim-sulfa (TMS). For enrofloxacin resistant strains, additional susceptibilities to macrophage-penetrating antimicrobials florfenicol, clarithromycin and rifampin were determined.[17] Breakpoints were based on CLSI interpretive standards[20] (NCCLS, 2004) and where CLSI interpretive standards were unavailable, breakpoints from the National Antimicrobial Resistant Monitoring System (NARMS) 2004 Annual Report were used.[21] *E. coli* strain ATCC 25922 was used as a quality control standard.

Statistical analysis

A Fisher's exact test was used to compare phylogroups, the number of antimicrobial resistant strains between GC and H, and clinical outcome with (a) susceptibility of *E. coli* strains to enrofloxacin and (b) prior treatment with enrofloxacin. Significance was set at $P<0.05$.

RESULTS

E. coli were observed in all GC (14/14), within the colonic mucosa and as multifocal clusters of bacteria within macrophages (Figure 3.1). Characterization of *E. coli* isolates by Triplex - and RAPD- PCR (Figure 3.2) yielded 23 *E. coli* strains from GC (1-3 per dog, median 2) and 34 from H (1-3 per dog, median 2) ($P>0.05$). The distribution of *E. coli* phylogroups was similar ($p=0.18$) in *E. coli* isolated from GC (5A, 7B1, 5B2, 6D) and H (2A, 10B1, 15B2, 7D). In the GC group, 3 dogs (Figure 3.2, lanes 5, 8 and 17) harbored a phylogroup B1 *E. coli* with identical banding patterns for RAPD 1283; subsequent analysis with RAPD primers 1254 and 1290 showed identical banding patterns in 2 of these 3 strains (Figure 3.2, lanes 8 and 17). In the H group, 2 dogs with *E. coli* in phylogroup A, and 4 with *E. coli* in phylogroup B2, had strains that were identical using all 3 RAPD primers. When comparing the GC and H groups, one strain from GC and H in phylogroup B1 (Figure 3.2, lane 26 GC, lane 28 H), and one from phylogroup B2 (Figure 3.2, lane 27 GC, lane 29 H) were identical on RAPD 1283, 1254 and 1290. A significantly greater number of GC-associated *E. coli* strains were resistant to ampicillin, amox-clav, cefoxitin, tetracycline, TMS, ciprofloxacin and chloramphenicol, when compared to strains from H (Table 3.1). Enrofloxacin resistant *E. coli* were isolated from 6/14 GC vs. 0/17 healthy ($p<0.001$). The susceptibilities of these 8 resistant strains to other fluoroquinolones and macrophage-penetrating antimicrobials with efficacy against *E. coli* [17] are shown in Table 3.2. Where resistance to enrofloxacin occurred, it was present in all *E. coli* strains isolated from an individual, and was associated with resistance to all other fluoroquinolones tested

(ciprofloxacin and marbofloxacin 8/8 strains, orbifloxacin 3/3 strains). Only 4 of 8 enrofloxacin resistant strains (in 2 individuals) were susceptible to chloramphenicol; 2 of 8 strains were susceptible to tetracycline or TMS; 1 of 8 was susceptible to clarithromycin, and none were susceptible to rifampin.

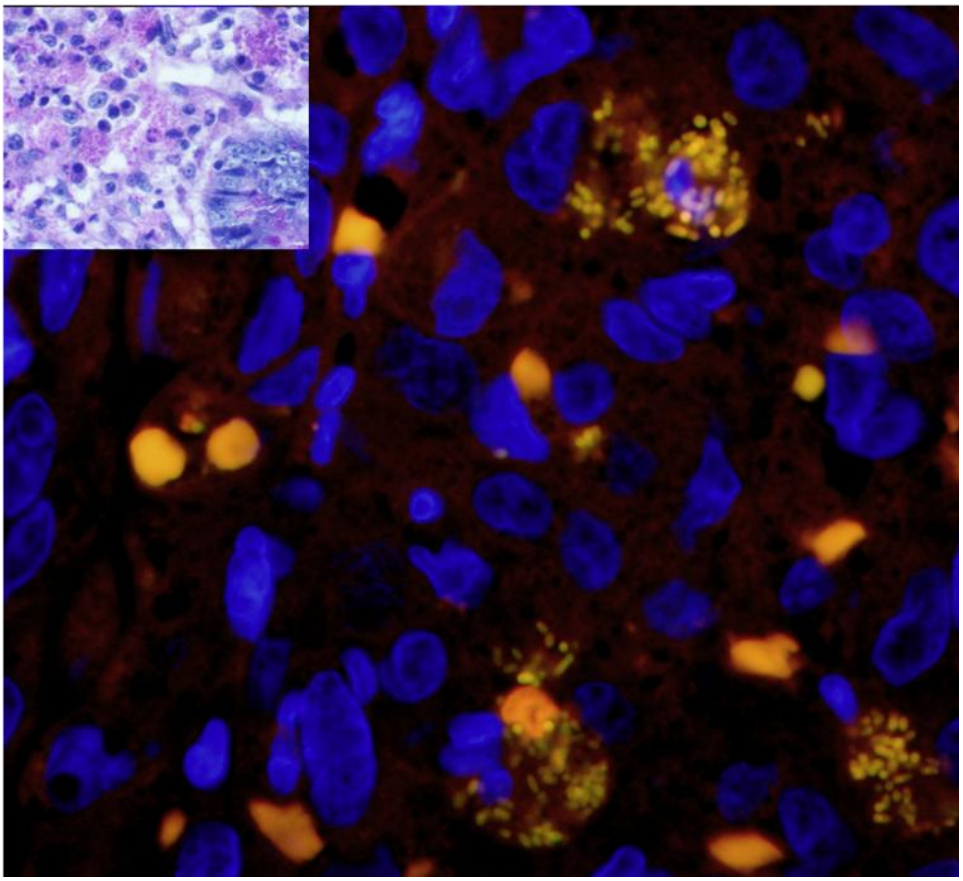

Figure 3.1: Fluorescence in situ hydridization (FISH) using *E. coli*-Cy3 (red) and eubacterial (EUB338-6FAM, green) probes, revealing multifocal clusters of intramucosal and intracellular *E. coli.* (x60). Inset – PAS stain showing many PAS positive macrophages (x40).

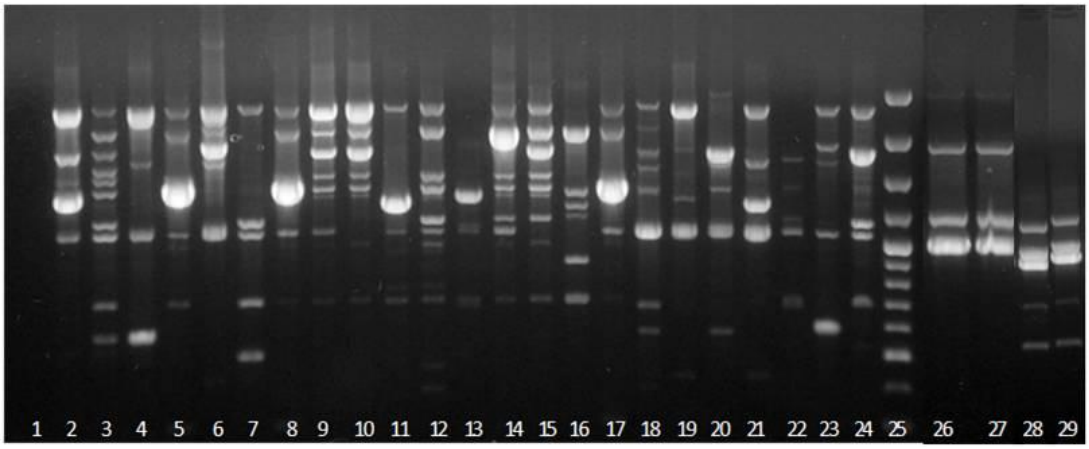

Figure 3.2: Genetic diversity of 23 GC-associated *E. coli,* evaluated by random amplified polymorphic DNA PCR (RAPD-PCR). Lanes 2–24: primer 1283, showing identical banding in lanes 5, 8, and 17 in 3 strains from 3 dogs. Lanes 26–29: RAPD-PCR primer 1254 on 2 isolates from GC (26, 28) and H (27, 29) with identical 1283 RAPD patterns. Lane 1: mastermix/primers/H20 control. Lane 25: 100 bp plus DNA ladder.

Table 3.1: Prevalence of antimicrobial resistance in *E. coli* strains isolated from 14 GC affected Boxer dogs and 17 healthy dogs. (Fisher Exact, GC/HUC vs H: *=P<0.05, **<0.01,***P<0.001).

	Resistant strains (%)		**Resistant individuals (%)**	
Antimicrobial	GC	Healthy	GC	Healthy
Amoxicillin-clavulanate	35*	8	57*	12
Ampicillin	49**	15	64**	18
Cefoxitin	30***	0	50***	0
Tetracycline	48*	18	64*	24
Trimethoprim-sulfa	44***	6	57**	7
Ciprofloxacin	35***	0	43**	0
Gentamicin	13	18	14	36
Chloramphenicol	17*	0	21	0

Table 3.2: Susceptibility of GC-associated enrofloxacin resistant *E. coli* to additional fluoroquinolones and macrophage-penetrating antimicrobials

Dog ID	**2**		**4**	**5**	**6**		**10**	**11**
***Response**	E		E	P	E		P	E
***E. coli* strain**	1	2	1	1	1	2	1	1
#Chloramphenicol	R	S	R	R	I	S	S	S
Florfenicol	I	I	R	R	R	I	I	S
Tetracycline	R	S	R	R	R	R	R	S
Trimethoprim-sulfa	R	R	R	R	S	R	S	R
Clarithromycin	R	R	R	R	R	R	R	S
Rifampin	R	R	R	R	R	R	R	R
Ciprofloxacin	R	R	R	R	R	R	R	R
Marbofloxacin	R	R	R	R	R	R	R	R

*E: euthanized due to disease, P: partial response

#R: resistant, S: susceptible, I: intermediate susceptibility

Most of the GC (11/14) had received empirical treatment with a number of antimicrobials (range 1-4, median 1), including metronidazole (11/14), enrofloxacin (8/14), tylosin (7/14), TMS (4/14), amoxicillin (3/14), ampicillin (1/14), and doxycycline (1/14) prior to colon biopsy. Empirical treatment with enrofloxacin was associated with the isolation of a resistant *E. coli* ($p<0.01$). Resistance to the antimicrobials given prior to biopsy was also documented in 3/4 dogs treated with TMS, 4/4 treated with ampicillin or amoxicillin, and 1/1 treated with doxycycline. There was no relationship of antimicrobial resistance to *E. coli* phylogroup (A, B1, B2 or D).

The time from a biopsy confirmed diagnosis of GC to last follow-up in the surviving dogs (n=10) was 27.5 months (median, range 18-78). 6 GC were classified as complete responders, 4 were partial responders, and 4 were non-responders that had been euthanized due to disease. In the 6 complete responders, all *E. coli* strains were susceptible to enrofloxacin, and only 2/6 had received enrofloxacin prior to definitive diagnosis. In contrast, the 4 non-responders harbored *E. coli* strains that were resistant to enrofloxacin and had received treatment with enrofloxacin prior to definitive diagnosis. Additional antimicrobial therapy in these dogs with various combinations of amikacin, neomycin enemas, erythromycin, cephalexin and amox-clav was unsuccessful. In the 4 partial responders, 2 were colonized with enrofloxacin susceptible *E. coli* and 2 with enrofloxacin resistant *E. coli*. Both of the partial responders with enrofloxacin resistant *E. coli* had received empirical treatment with enrofloxacin prior to biopsy. The owners of the partial responders reported that enrofloxacin was associated with a reduction in severity and frequency of clinical signs, and had employed intermittent 2 to 4 week treatments with enrofloxacin (3 dogs) or continuous enrofloxacin (1 dog).
To summarise, 6/6 complete responders harbored enrofloxacin susceptible *E. coli*, whereas 2/4 partial responders and 4/4 (euthanized) non-responders harbored enrofloxacin resistant strains. Clinical response (complete vs. partial responders + euthanized) was negatively impacted by resistance of mucosal *E. coli* to enrofloxacin, and treatment with enrofloxacin prior to definitive diagnosis ($p<0.01$).

DISCUSSION

Four independent studies have documented dramatic clinical responses of Boxer dogs with GC to antimicrobial regimens containing enrofloxacin,[4-7] and clinical and histological remission is associated with the eradication of invasive *E. coli*, typically within colonic macrophages.[4, 5] These findings have led to GC in Boxers being considered a breed-specific defect in mucosal immunity that enables opportunistic invasion by resident *E. coli*, [5] and enrofloxacin being regarded as the treatment of choice, with a positive clinical response reported in 19/20 dogs.[4-7] The lack of clinical response to enrofloxacin in the only non-responder reported to date was associated with persistence of intramucosal *E.coli*, the isolation of enrofloxacin resistant *E. coli,* lack of response to alternative antimicrobials, and eventually euthanasia.[5]

The present study was undertaken to inform the treatment of GC by determining the antimicrobial susceptibility profiles of *E. coli* associated with GC. We found that over 50% of GC harbored mucosal *E. coli* that were resistant to one or more non-fluoroquinolone antimicrobial, and resistance to fluoroquinolones (including enrofloxacin) was present in an alarming 43%. These observations suggest that antimicrobial resistance in GC-associated *E. coli* may have been selected for as a consequence of the growing use of fluoroquinolones in human and veterinary medicine, though we did not uncover any relationship of resistance to specific *E. coli* phylogroups.[22-25] It is also possible that invasive *E. coli* in GC affected Boxers have acquired resistance through prior treatment, as supported by the correlation between enrofloxacin resistance and empirical therapy. The majority of *E. coli* strains isolated from GC and H were diverse in overall genotype with minimal overlap

between H and GC. However, it is interesting to note that 2 GC dogs from different geographical locations harbored phylogroup B1 strains with identical RAPD-PCR patterns with 3 different RAPD primers. The absence of clustering by phylogroup and overall genotype suggests that no single clonal group is responsible for mucosal invasion in GC. In-depth analysis of genotype (virulence and phylogeny) and phenotype of GC and H *E. coli* is underway to try to identify the microbial characteristics associated with invasion of, and persistence within, the colonic mucosa.

Enrofloxacin resistance was significantly associated with clinical outcome, and it is a salient observation that complete remission of disease occurred only in the dogs that harbored enrofloxacin susceptible *E. coli*, with partial response or euthanasia resulting in all 6 GC with resistant strains. Due to the retrospective nature of collecting clinical details in this study, we were not able to determine precisely the dose, frequency and duration of enrofloxacin therapy prior to colonic biopsy of GC. In a prior study we found that enrofloxacin at 7mg/kg PO for at least 6 weeks was associated with a good clinical response, and we speculate that short-term empirical treatment may result in clinical improvement (often within one to 2 weeks[5]) that leads to cessation of therapy before complete eradication of *E. coli* and the emergence of resistant strains. The potential adverse effects of enrofloxacin on cartilage development in skeletally immature Boxer dogs may additionally influence practitioners to avoid treatment beyond clinical improvement. Prospective evaluation of the impact of antimicrobial therapy on *E. coli* isolated from GC affected Boxers is required to clarify these issues.

Given the high frequency of antimicrobial resistance in GC, we sought to identify alternative antimicrobials with efficacy against GC associated *E. coli*. The lack of clinical response of dogs that were unresponsive to enrofloxacin to antimicrobials with efficacy against their *E. coli* strains *in vitro* (e.g. amikacin, amox-clav, neomycin) suggests that factors such as drug distribution impact the eradication of mucosally invasive *E. coli*. Support for this is provided by a recent study of CD-associated *E. coli* (AIEC), where the ability of an antimicrobial to kill *E.coli* within macrophages was determined by a combination of antimicrobial susceptibility and the ability to accumulate intracellularly[17]. For example, ampicillin was effective against CD-associated *E. coli* in culture, but ineffective against bacteria within cultured macrophages.[17] The antimicrobials with best efficacy for intracellular killing were ciprofloxacin, 99.5%; rifampin, 85.1%; tetracycline, 62.8%; clarithromycin, 62.1% sulfamethoxazole, 61.3%; TMS, 56.3% and azithromycin, 41.0%.[17]

In the present study, the most effective antimicrobials against GC associated *E. coli* on the basis of MIC testing were amikacin (23/23 strains and 14/14 dogs susceptible) and chloramphenicol (19/23 strains and 11/14 dogs susceptible). The aminoglycosides are ineffective against facultative intracellular pathogens such as *Shigella* spp., *Salmonella* spp. during their intracellular growth phase, because of their inability to effectively penetrate mammalian cells.[26] Amikacin is therefore unlikely to be an effective agent for elimination of intracellular *E. coli* in GC. Chloramphenicol is small and lipid soluble, penetrates effectively into most tissues, and may therefore be an effective alternative in fluoroquinolone resistant cases. Chloramphenicol has

also been associated with positive treatment responses in 6/8 GC affected Boxer dogs.[1] However, in the present study 50% of the fluoroquinolone resistant *E. coli* strains were also resistant to chloramphenicol, and only 2/6 GC with enrofloxacin resistant *E. coli* harbored chloramphenicol susceptible strains. The potential adverse effects of chloramphenicol (aplastic anemia) have also tended to restrict its use in dogs. Florfenicol is a newer derivative of chloramphenicol that has not been associated with aplastic anemia, is almost completely absorbed after oral administration in dogs, and is not subject to the action of acetyltransferase, an enzyme used by bacteria to develop resistance to chloramphenicol.[27] In the present study we observed intermediate susceptibility of GC associated *E. coli* to florfenicol that were susceptible to chloramphenicol, but it is unclear if this is a consequence of the breakpoints used for interpretation of MIC for florfenicol, which have not been determined for canine isolates (the same is true for chloramphenicol).

Interestingly, in contrast to CD-associated *E. coli,*[17] isolates from GC were frequently resistant to rifampin, clarithromycin, TMS, and tetracycline, which severely limits the selection of alternative antimicrobials in enrofloxacin resistant GC. We speculate however that the *in vivo* activity and potential for synergism in bacterial killing may be underestimated by *in vitro* testing of individual antimicrobials. In this respect, synergistic responses to combination therapy with multiple antimicrobials, e.g. ciprofloxacin, tetracycline and trimethoprim have been reported in CD-associated *E. coli*.[17] This multidrug approach may also be helpful in enrofloxacin resistant GC but remains to be evaluated.

In conclusion, the findings of this study indicate that antimicrobial resistance is common in *E. coli* associated with GC. Resistance to enrofloxacin is associated with a poor clinical outcome, and empirical antimicrobial treatment prior to definitive diagnosis. To optimize outcome it would seem preferable to perform colonic biopsy to achieve a definitive diagnosis (histopathology and FISH), and isolation and susceptibility testing of colonic mucosal *E. coli* to optimize treatment prior to administration of antimicrobials.

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CHAPTER 4

Genome-Wide Analysis of Granulomatous Colitis

in the Boxer Dog.

ABSTRACT

Granulomatous colitis (GC), also known as histiocytic ulcerative colitis (HUC) is a breed-specific inflammatory bowel disease (IBD) of young Boxer dogs. Culture-independent molecular analysis has recently uncovered a correlation between GC and mucosally invasive *E.coli*. Eradication of intramucosal *E. coli* using antimicrobials (typically enrofloxacin) is associated with clinical and histologic disease remission. The breed specificity and disease characteristics suggest a heritable immunologic genetic susceptibility in the Boxer dog, which we sought to investigate using a genome-wide association scan (GWAS).

The GC phenotype was confirmed in 17 Boxer dogs (9F, 8M) by presence on histology of PAS+ macrophages. Mucosally invasive *E.coli* were detected by FISH analysis in all dogs. The control group consisted of 11 Boxer dogs (4M, 7F), >8 years old, with no history of gastrointestinal disease. Genomic DNA was harvested from blood samples collected by venipuncture into sodium EDTA, and a GWAS was performed using the Canine Affymetrix v2.0 single nucleotide polymorphism (SNP) array. Genotypes were analyzed using a MAGIC algorithm developed specifically for multiple locus GWAS analysis, enabling statistical comparisons of case and control populations (Fisher exact).

GC-associated SNPs (rs8955971, p=4.3e-06 and rs24450648, p=9.12e-05) in the gene encoding neutrophil cytosolic factor 2 (NCF2, chromosome 7: 19,875,317-19,904,798). The product of NCF2 is a cytosolic subunit of the multi-protein complex NADPH oxidase, found in phagocytes. The NADPH complex is an essential component of bacterial killing, required for production of the 'respiratory burst' by delivery of reactive oxygen species to the phagosome. Dysfunction of the NADPH complex results in an ineffective respiratory burst and inability to eliminate intracellular pathogens, particularly catalase producing bacteria and fungi. Mutations in NCF2, as well as 3 other subunits (NCF1, CYBBa, CYBBb), are known to cause chronic granulomatous disease (CGD) in people, a disease complex comprising immunodeficiency disorders and predisposition to chronic infections. Patients with CGD may develop colitis characterized by 'exuberant, pigment laden macrophages' (Schäppi, Pediatr Gastroenterol Nutr '03;36) and are frequently maintained on chronic antibiotic therapy, though the specific bacteria involved are undefined. A recent GWAS in human IBD has also identified Crohn's disease-associated SNPs in the gene encoding an additional NADPH oxidase complex subunit, NCF4.

In summary, we have identified NCF2 as a candidate gene for GCB, which guides further disease mapping and phenotypic characterization by neutrophil function testing. The known causative NCF2 mutations in human CGD add weight to this finding, and our results suggest a potential role for *E. coli* in the pathogenesis of CGD colitis in people.

INTRODUCTION

Granulomatous colitis (GC) is an uncommon type of inflammatory bowel disease (IBD), seen predominantly in Boxer dogs under 4 years of age.[1-4] There are sporadic reports of granulomatous colitis (GC) in other dog breeds,[5] particularly young French Bulldogs[6] (and author observation). Affected dogs typically present with signs of colitis, hematochezia and weight loss, progressing to cachexia in severe cases.[1, 7-9] Grossly, the colon mucosa has a friable, ulcerated and cobblestone appearance. Diagnosis is based on colonic histology, and typified by large numbers of periodic acid-Schiff (PAS)-positive macrophages, accompanied by lymphocytes, plasma cells, eosinophils, epithelial ulceration and loss of goblet cells. The PAS positive macrophages are a unique and pathognomonic feature of GC, not seen in any other type of canine IBD.

Because of histologic similarities to Whipple's disease in people, caused by the bacteria *Tropheryma whipplei,*[10,11] and following clusters of disease which responded to chloramphenicol,[1] an infectious etiology was initially suspected in GC. Thus, early studies focused on the search for a GC-associated pathogen, e.g. *Chlamydia*,[12] *Mycoplasma*,[13] but were unsuccessful and a primary immune-mediated pathogenesis was presumed.[7,14] The mainstay of treatment for GC for many years was immunosuppression, but responses to treatment were generally poor, frequently resulting in euthanasia. GC became considered a severe, incurable immune-mediated disease.[4-7,14]

The hunt for an infectious cause of GC was re-ignited by reports of long-term remission in dogs treated with enrofloxacin.[3,15,16,17] More recent work using fluorescence in situ hybridization (FISH) has demonstrated a firm

association with *E. coli,* and intramucosal and intracellular invasion by *E coli* is now documented in the majority of cases.[17-19] GC-associated *E. coli* have been shown to lack genes typically associated with virulence, and were able to invade epithelial cells and persist within macrophages.[17] This pathogen-like behavior is similar to a newly identified *E. coli* pathotype, the 'Adherent and Invasive *E. coli* (AIEC),' that are increasingly associated with Crohn's disease (CD) in people.[17,20-22]

Because GC is breed specific and relatively rare, an autosomal recessive genetic defect involving the immune system that confers susceptibility to *E. coli* invasion is suspected. In people and dogs, genome wide association analysis (GWAS) has been shown to be an effective approach in identifying gene polymorphisms associated with disease phenotypes. Dogs are particularly well suited to GWAS due to low levels of intrabreed variation, long haplotype blocks (0.5-1Mb), and extensive linkage dysequilibrium. Thus, fewer, less dense markers are required for mapping. In a recent canine GWAS, it was estimated that just 15,000 SNPs could define a locus for a recessive trait. These authors showed that 27,000 SNPs mapped Mendelian traits in 10 affected and 10 controls for the Rhodesian Ridgeback ridge and white spotting locus.[23]

The aim of this study was to explore the genetic basis of GC by GWAS. Identification of candidate genes in such a well-defined model clearly holds great potential to advance understanding of the interplay between luminal pathogens and the host response in human and canine IBD. This in turn may facilitate the development of specific therapeutic targets in preference to current empirical management strategies.

METHODS

Animals: Affected dogs were sampled as part of their diagnostic evaluation, therefore approval by the Institutional Animal Care and Use Committee was not required. Pedigree information is rarely available for these dogs because they often originate from Animal Shelters due to the severity of their clinical signs. Healthy Boxer dogs were sampled with informed client consent.

Sample collection: Blood was collected into EDTA from GC-affected Boxer dogs. Controls consisted of 11 healthy Boxer dogs > 8 yrs old, with no history of chronic gastrointestinal (GI) signs. DNA was extracted by a modified phenol chloroform technique and archived at -80 ºC.

Clinical diagnosis of *E. coli*-associated GC: The GC phenotype was established by excluding other causes of chronic diarrhea (by e.g. fecal parasitology, culture, imaging studies, Giardia/Cryptosporidium ELISA) and finding granulomatous inflammation and PAS+ macrophages in colon biopsies. Intramucosal and intracellular *E. coli* invasion was demonstrated using FISH on formalin-fixed, paraffin-embedded colonic mucosal biopsies, with eubacterial (EUB338-6FAM, GCTGCCTCCCGTAGGAGT) and *E. coli*-specific 16S rDNA probes (*E. coli*-Cy3, GCAAAGGTATTAACTTTACTCCC).[17]

Genome-wide association study: Dogs were genotyped using Affymetrix Version 2.0 Canine single nucleotide polymorphism (SNP) chips and the standard Affymetrix GeneChip Mapping 250K Sty Assay protocol (Affymetrix Inc., Santa Clara, CA), using 250ng genomic DNA. Genotypes were called using the MAGIC algorithm,[24] on batched sets of the Affymetrix probe results files (CEL files). All calls were combined into one dataset and single marker

chi-squared association, model-based Fisher association, and haplotype association was performed using gPLINK. Genotype calls in the regions of interest were assembled into haplotypes to identify the minimal linkage disequilibrium (LD) interval.

Neutrophil function test: Following identification of NCF2 as a candidate gene, a flow cytometry-based test of neutrophil function was developed, based on previously used methodology.[25] Blood samples for neutrophil function were obtained by jugular venipuncture into EDTA tubes, transported on ice and analyzed within 24 hours of collection. Red blood cells were lysed using 0.84% ammonium chloride, and the cell pellet was washed 3 times in magnesium-free Hanks balanced salt solution (HBSS, Gibco, Grand Island, NY, USA) using centrifugation at 150g at 4ºC, for 10 min. Cell pellets were resuspended in 1 ml HBSS and kept on ice until assay. A leukocyte count was manually performed using a Neubauer cell counting chamber. Approximately 1-2x10^6 leukocytes were suspended in 200 ul HBSS, and 100 ul of Dihydrorhodamine-1,2,3 (Molecular Probes Inc., Eugene, OR, USA) was added to resting and stimulated control and sample tubes, and incubated at 37ºC for 5 minutes. Neutrophils were stimulated by the addition of phorbol myristate acetate (PMA), followed by incubation at 37ºC for 15 minutes. Samples were immediately analyzed on a BD FACSCalibur flow cytometer, and data analysis was performed using FlowJo software (Ashland, OR). The following controls and samples were included:

1. Cell suspension alone, at 4ºC and at 37ºC ('resting' tubes).
2. Cells + DHR at 4ºC and 37ºC ('resting' tubes)
3. Cells + DHR + PMA at 4ºC and 37ºC (stimulated samples).

RESULTS

Clinical phenotypes

GC was confirmed in 17 Boxer dogs (9F, 8M, median age 1 yr) by finding PAS+ macrophages on colon histology. FISH revealed intramucosal *E. coli* invasion in all affected dogs (Figures 4.1 and 4.2). Controls consisted of 11 Boxer dogs >8yrs of age (4M, 7F), with no history of GI signs.

GWAS analysis.

Figure 4.3 shows a Manhattan plot of logged p values for the GWAS. Analysis of the reciprocal range (1/range) generated from two GWAS was used to narrow down chromosomes of highest interest. The 1/range statistic is a measure of the consistency of the p-value, and the information content. That is, a high value derives from a very consistent finding, with a low difference or range between the 2 estimates. The combination of a high average - Log10p, and a high value for 1/range ranked chromosomes 1, 7, and 24 as the most worthy of further pursuit (Table 4.1).

Table 4.1: (-Log10p) for chromosomes with the highest p values

Chr	Avg (-Log10p)	1/range
1	**5.180**	**6.20**
2	5.463	0.70
5	4.777	2.65
7	**5.403**	**13.61**
9	5.761	0.36
16	5.013	0.96
18	4.855	1.13
24	**6.401**	**14.68**
X	4.317	1.69

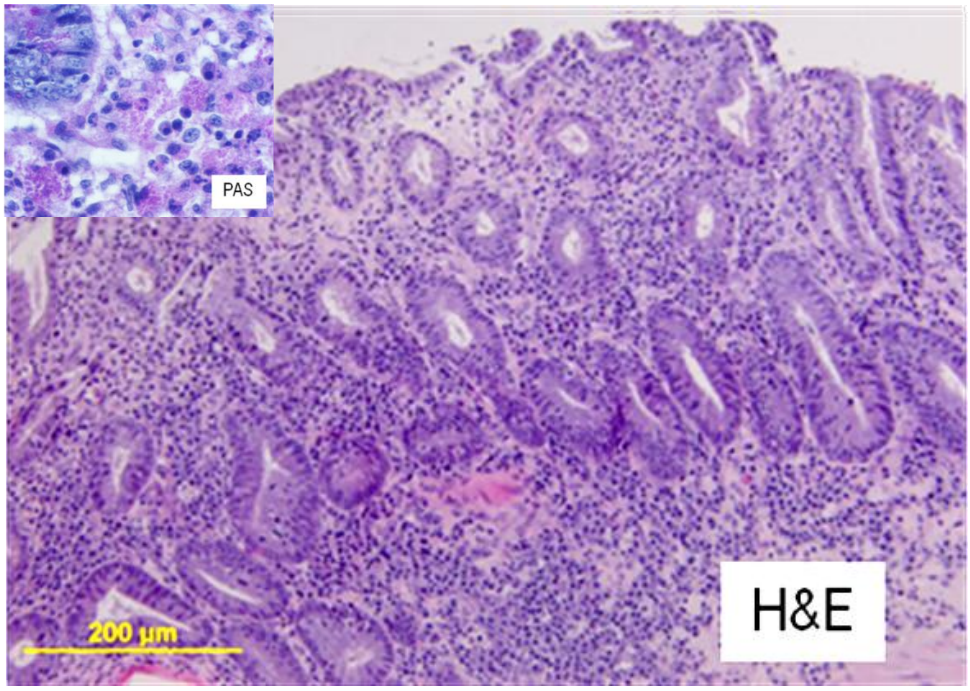


Figure 4.1: Colonic mucosa in GC is ulcerated and inflamed, with loss of glandular structure and (inset) mucosal infiltration with PAS+ macrophages (x40).

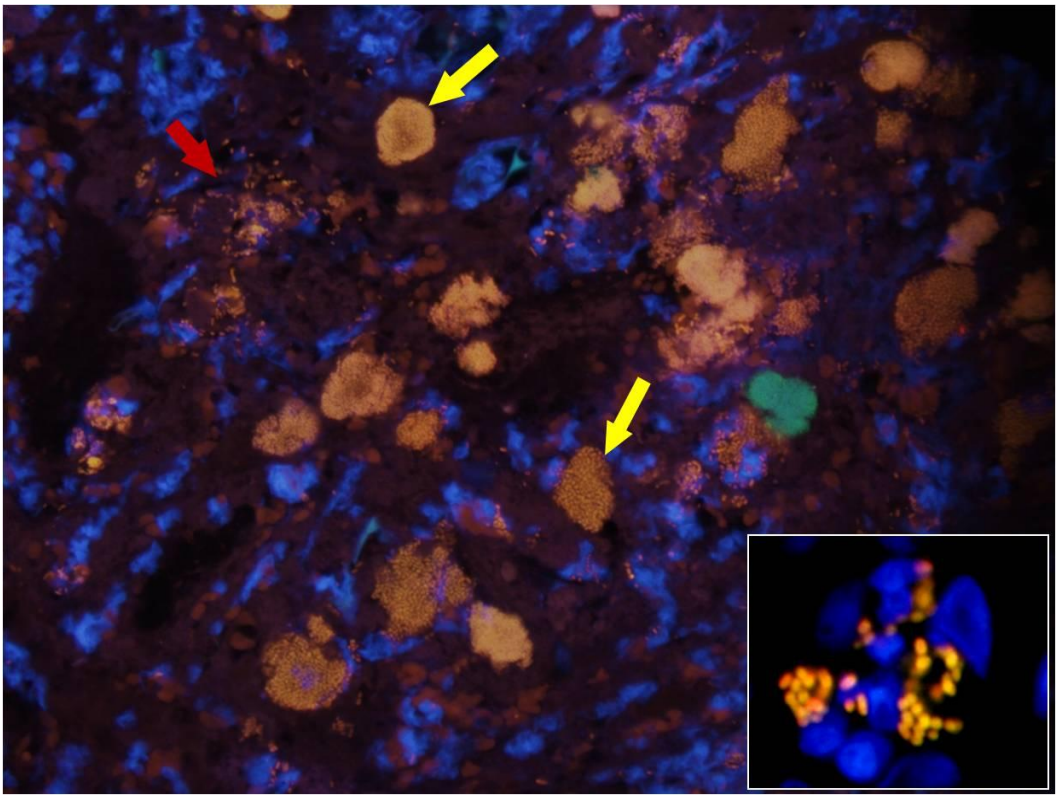
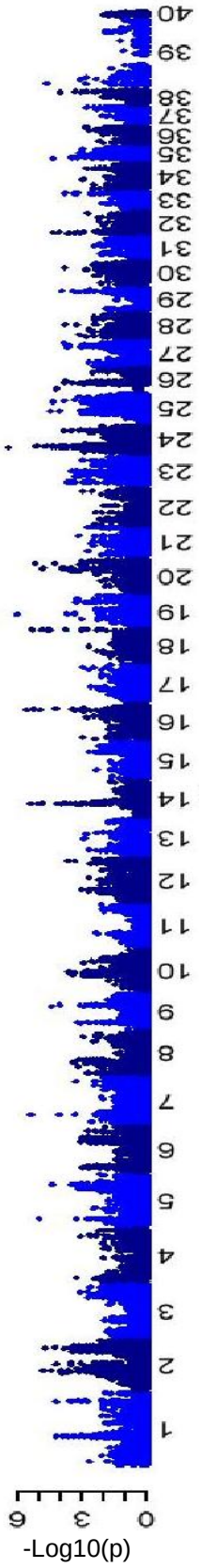


Figure 4.2: FISH image (x40) of GCB colon mucosa showing typical clusters of *E. coli* within the mucosa (red arrow) and intracellularly with macrophages (yellow arrows) **Inset** (x200)**:** invasive *E. coli* within a macrophage. *E. coli*-Cy3 probe (red) with non-EUB3386FAM (green) and DAPI (nuclei in blue).

Figure 4.3: Manhattan plot showing significance of association of all SNPs in the GWAS Fisher's exact test with GC and control phenotypes. SNPs are plotted on the x-axis according to their position on each chromosome against association with GC on the y-axis (shown as -log10 p value).


40
39
38
37
36
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32
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21
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
6
3
0
-Log10(p)

GC-associated SNPS are located in NCF2

GC-associated chromosome 7 SNPs were located 800bp apart, in an intron of the gene encoding neutrophil cytosolic factor 2 (NCF2). This gene encodes a cytosolic subunit, p67^{phox}, of the multi-protein complex NADPH oxidase.[26, 27] Within phagocytes, NADPH oxidase has a crucial role in innate immunity by reducing molecular oxygen to superoxide (Figure 4.4), giving rise to numerous toxic reactive oxygen species (ROS). ROS are used as microbicidal agents against pathogens in the 'respiratory burst' generated by phagocytic cells[28, 29]. An ineffective respiratory burst results in compromised ability to eliminate intracellular pathogens, particularly catalase-producing bacteria and fungi.[26, 30] Mutations in NCF2 in people are known to cause chronic granulomatous disease (CGD), a disease complex comprising immunodeficiency disorders and predisposition to chronic infections.[26, 29, 31] Patients with CGD can develop colitis with striking histologic similarities to GC, including PAS+ macrophages.[32,33]

Table 4.2: genotypes of GC-associated SNPs on chromosome 7, in 17 GC-affected and 11 healthy Boxer dogs: 19895486 (rs8955791), 19896293 (rs24450648) are located within an intron of the gene, and 20506915 is further upstream of the gene.

SNP	GC-AFFECTED (n=17)																	CONTROL (n=11)										p (Fisher's)
Chr 7: 19895486	1	1	2	1	1	1	2	2	2	2	2	2	1	2	1	1	2	0	0	0	1	1	0	1	0	0	0	-1	4.30E-06
Chr 7: 19896293	1	2	2	2	1	1	2	2	2	2	2	2	1	2	2	1	2	0	0	0	1	-1	0	2	0	0	0	1	9.12E-05
Chr 7: 20506915	1	1	2	-1	2	-1	2	2	0	2	2	2	2	2	0	-1	2	0	0	0	-1	-1	0	-1	0	0	0	1	3.63E-06

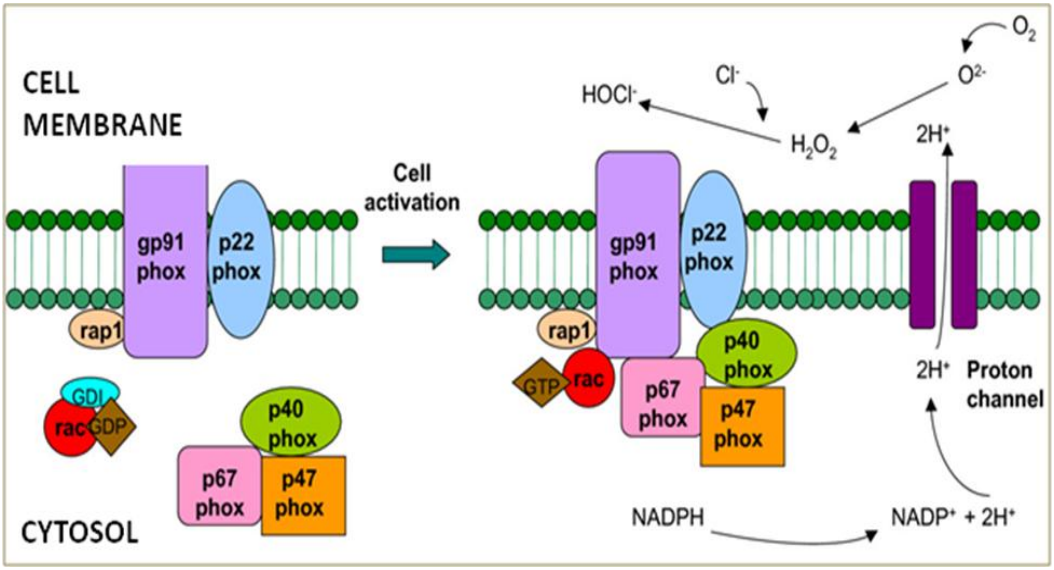


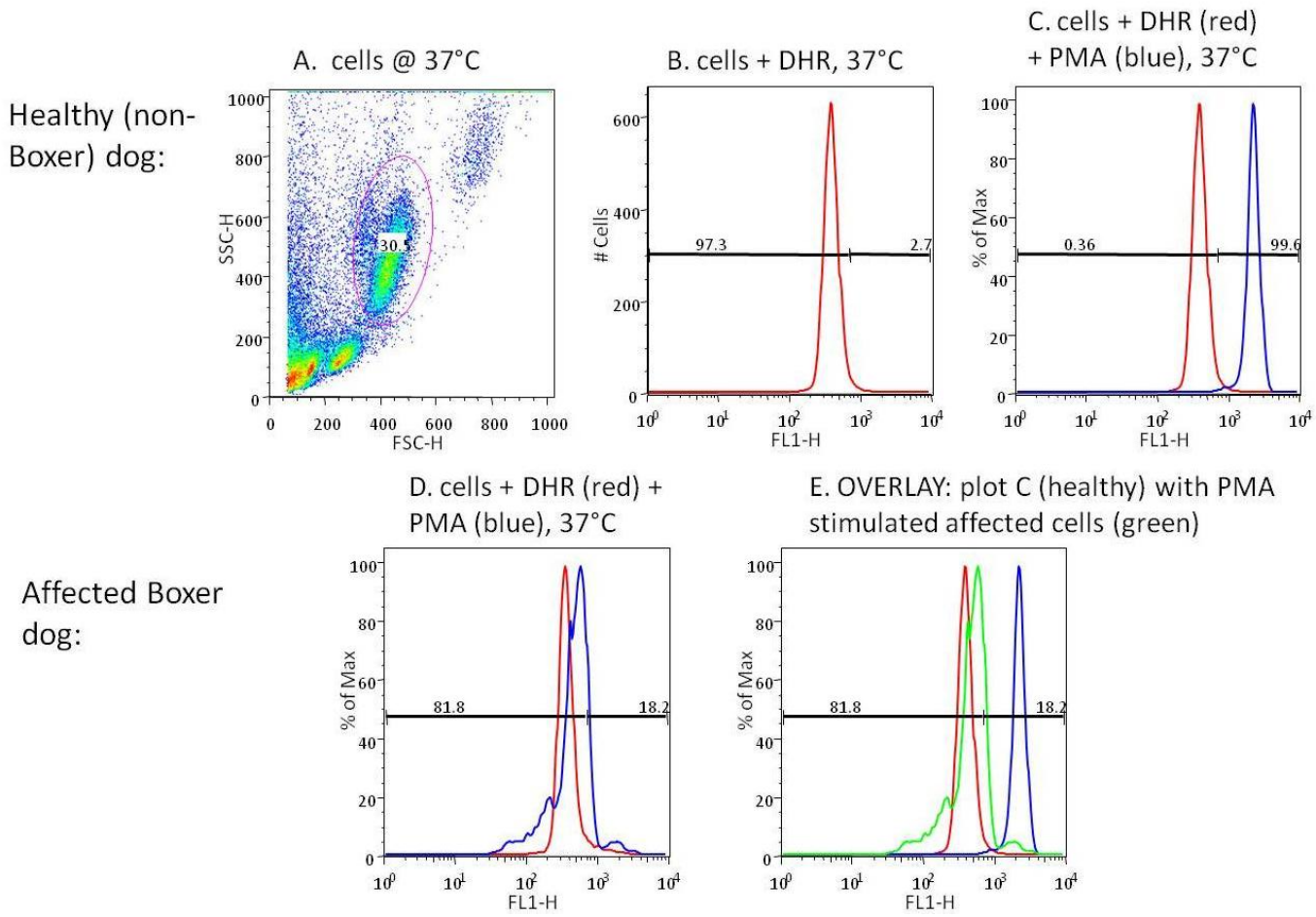
Figure 4.4: the NADPH oxidase complex within phagocytes. On cellular activation, the cytosolic components p47phox and p67phox are phosphorylated and bind tightly together. In association with p40phox and rac, these proteins combine with the cytochrome complex (gp91phox and p22phox) to form the intact NADPH oxidase. An electron is taken from NADPH and donated to molecular oxygen, leading to the formation of superoxide. In the presence of superoxide dismutase, this is converted to hydrogen peroxide, which, in the presence of myeloperoxidase and chlorine in the phagosome, is converted to bleach. This is essential for bacterial killing.

Neutrophil function testing supports an NCF2 mutation in GC.

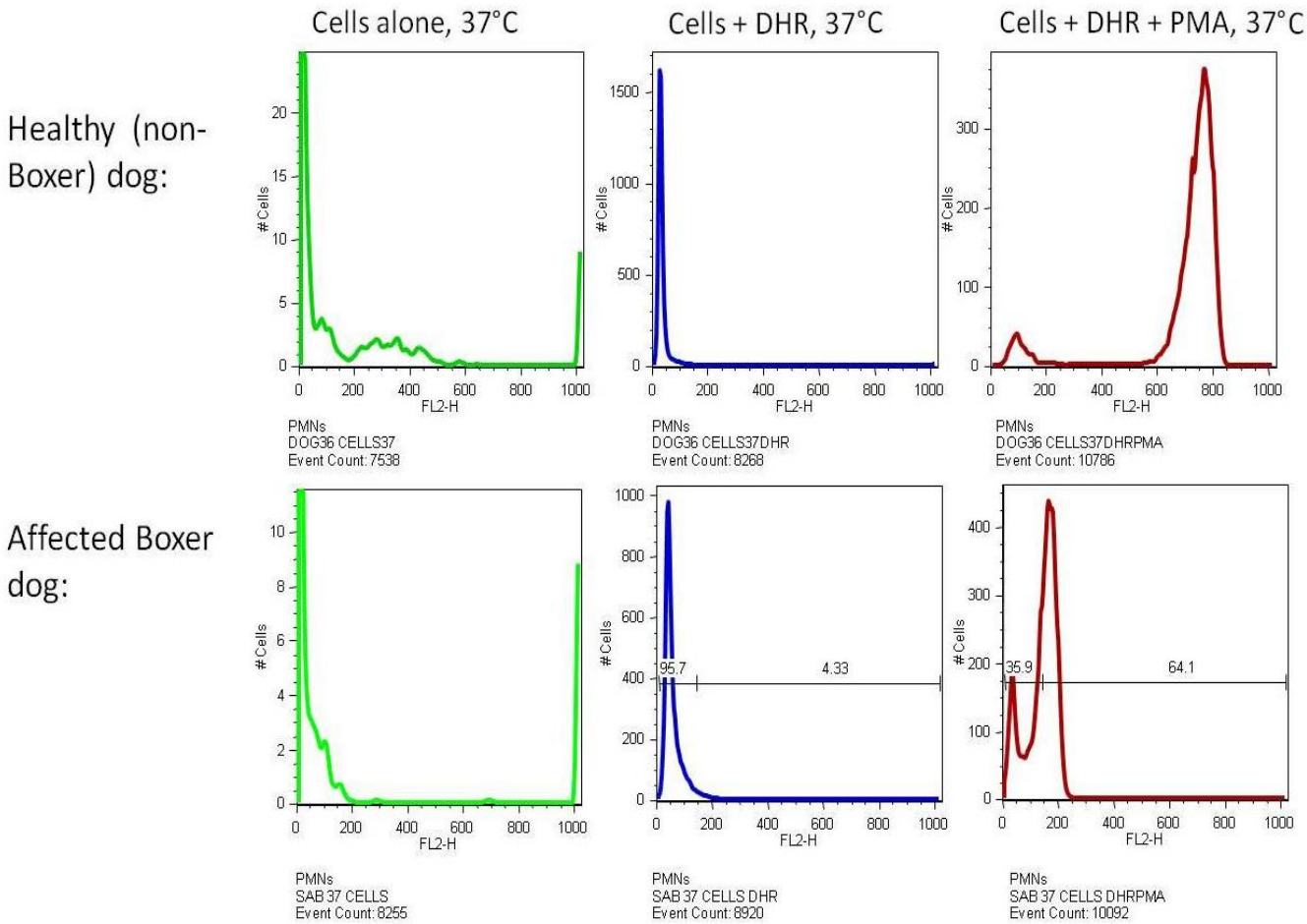
After discovering NCF2-associated SNPs, we further evaluated the phenotype of affected Boxer dogs by assessment of neutrophil function. This is the screening test for CGD in people.[34] It is performed by testing the neutrophil respiratory burst, which is a series of reactions in which superoxide ions are generated by the NADPH oxidase, and metabolized to reactive oxygen species. The end products of this process are highly toxic to ingested microorganisms, and the process is well recognized as an essential part of innate immunity. Flow cytometric assay of neutrophil function uses dihydrorhodamine 123 (DHR) as a substrate for the respiratory burst in isolated neutrophils. In stimulated neutrophils, DHR is oxidized to rhodamine by hydrogen peroxide. Rhodamine, which is fluorescent, is then detected and measured in a flow cytometer. The oxidation of DHR is dependent on NADPH oxidase, and the assay is a measure of the critical early stages of the neutrophil respiratory burst. In 2 GC-affected boxer dogs, we identified severely compromised neutrophil function relative to healthy controls (Figure 4.5).

Figure 4.5: Flow cytometric analysis of peripheral neutrophils from 2 GC-affected dogs compared to healthy dogs. **(a)** Graphs A-C, control dog (top line) showing Dihydrhodamine (DHR) stained neutrophils red (B) and post-phorbol myristate acetate (PMA) stimulation (C, blue) - >99% of control dog neutrophils underwent a superoxide burst. In the GC-affected dog (bottom line) <20% of neutrophils underwent the superoxide burst (D) and (E), curve overlay for comparison of healthy (red, blue) and affected (green) overlay of PMA stimulated cells from affected dog #1 (green) with control dog (blue). **(b)** Oxidation of DHR after PMA stimulation is significantly impaired in the GC-affected dog #2 (bottom line), compared to the control dog (top line).

(a).


Healthy (non-Boxer) dog:
A. cells @ 37°C
B. cells + DHR, 37°C
C. cells + DHR (red) + PMA (blue), 37°C
SSC-H
FSC-H
30.5
Cells
97.3
2.7
FL1-H
% of Max
0.36
99.6
Affected Boxer dog:
D. cells + DHR (red) + PMA (blue), 37°C
E. OVERLAY: plot C (healthy) with PMA stimulated affected cells (green)
81.8
18.2

(b).


Cells alone, 37°C
Cells + DHR, 37°C
Cells + DHR + PMA, 37°C
Healthy (non-Boxer) dog:
Affected Boxer dog:
#Cells
FL2-H
PMNs
DOG36 CELLS37
Event Count: 7538
PMNs
DOG36 CELLS37DHR
Event Count: 8268
PMNs
DOG36 CELLS37DHRPMA
Event Count: 10786
PMNs
SAB 37 CELLS
Event Count: 8255
PMNs
SAB 37 CELLS DHR
Event Count: 8920
PMNs
SAB 37 CELLS DHRPMA
Event Count: 10092
95.7
4.33
35.9
64.1

GC-associated SNPs in chromosomes 1 and 24.

Although NCF2 is the most promising candidate gene, statistically stronger disease-associated SNPs were located in chromosomes 1 and 24 (Tables 4.3 and 4.4 respectively). No obvious GC-associated candidate genes are present in this region of chromosome 24, which may indicate a previously unmapped gene. There are however several additional candidate genes on chromosome 1. GC-associated SNPs are located in and around the gene encoding the receptor for insulin-like growth factor 2 (IGF-2R, Chr 1 52203896-52304596), also called the cation-independent mannose-6-phosphate (M6P) receptor, (LD 583 Kb).[35] The M6P binding site of this receptor mediates lysosomal trafficking, TGF-β activation and cell-mediated cytotoxic cell death.[35] Thus, defects in the M6P/IGF-2R may be expected to impact T-cell mediated immunity, as well as the intracellular trafficking of lysosomal enzymes and bacterial killing.[35] A human homolog close to this region is the gene SLC22A2, which encodes an organic cation transporter gene cluster (OCTN1-2). Variants of this gene confer susceptibility to IBD in people, but have not yet been associated with IBD in dogs.[36]

Table 4.3: Haplotype for GC-associated chromosome 1 SNPs

SNP	GC-AFFECTED (n=17)																	CONTROL (n=11)										p (Fisher's)
chr1.52117003	1	1	1	2	2	1	1	1	1	1	1	2	1	2	1	1	2	1	0	0	0	1	0	0	0	0	1	0	0	2.20E-05
chr1.52122055	1	1	1	2	2	1	1	1	1	1	1	2	1	2	1	1	2	1	0	0	0	1	0	0	0	0	1	0	0	2.20E-05
chr1.52150417	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	2	2	2	-1	2	2	2	2	1	2	2	7.96E-06
chr1.52186288	1	1	1	2	2	1	1	1	1	1	1	1	1	2	1	1	1	1	0	0	0	1	0	0	0	0	1	0	0	2.20E-05
chr1.52591950	1	1	1	2	2	1	1	1	1	1	1	1	1	2	1	1	1	1	0	1	0	1	0	-1	0	0	1	0	0	2.79E-04
chr1.52856943	1	1	1	1	2	1	1	1	1	1	1	1	1	2	0	1	1	1	0	0	0	1	0	0	0	0	1	0	0	1.90E-04
chr1.52857620	1	1	1	1	2	1	1	1	1	1	1	1	1	2	0	1	1	1	0	#	0	-1	-1	-1	0	-1	1	0	0	1.90E-04
chr1.52876044	1	1	1	1	2	1	1	1	1	1	1	1	1	2	0	1	1	1	0	0	0	1	0	0	0	0	1	0	0	1.90E-04

Table 4.4: Haplotype for GC-associated chromosome 24 SNPs

SNP	GC-AFFECTED (n=17)																	CONTROL (n=11)										p (Fisher's)
chr24.14896681	2	2	2	2	2	2	2	2	2	1	2	2	2	2	2	2	1	1	2	1	2	0	1	0	1	1	1	1	3.28E-05
chr24.14901976	2	2	2	2	2	2	2	2	2	1	2	2	2	2	2	2	1	1	2	1	2	0	1	0	1	1	1	1	3.28E-05
chr24.15017116	2	2	2	2	2	2	2	2	2	1	2	2	2	2	2	2	1	1	2	1	2	-1	-1	0	1	1	1	1	1.65E-04
chr24.15647663	0	2	2	1	1	1	0	0	1	1	0	0	-1	1	1	-	1	2	2	2	1	2	2	2	2	2	2	-	-1	2.39E-04
chr24.15837274	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	0	1	-	0	1	0	1	1	-	2	2.13E-06
chr24.15905629	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	2	1	0	2	1	2	1	1	1	1	2.43E-05

DISCUSSION

Here we sought to investigate the genetic basis of GC by performing a genome-wide association scan (GWAS), and discovered GC-associated SNPs in the gene encoding NCF2. Mutations in NCF2 in people cause an immunodeficiency syndrome known as Chronic Granulomatous Disease (CGD). Dysfunction of NADPH oxidase in CGD results in impaired bacterial killing by phagocytes, and increased susceptibility to infections. Remarkably, approximately 50% of people with CGD also develop colitis with strikingly similar histologic features to GC of Boxer dogs, including the pathognomic finding of PAS+ macrophages.[31-33] The identification of NCF2-associated SNPs in Boxer dog GC may explain the impaired killing of intramucosal *E. coli* and PAS+ macrophages associated with this disease. Although, statistically speaking, the NCF2 SNPs do not show the strongest disease association, from a comparative and biologic standpoint, this is by far the most promising candidate gene. It is also notable that a recent GWAS in human IBD has identified CD-associated SNPs in the gene encoding another NADPH oxidase complex subunit, NCF4.[37]

CGD is a single disease with 4 genetic etiologies, reflecting the 4 protein subunits that make up the NADPH oxidase enzyme complex. NCF2 encodes one of 2 cytosolic subunits (p67^{phox}), the other unit being p47^{phox}, encoded by NCF1. The 2 membrane-bound subunits are gp91^{phox} and gp22^{phox} encoded by cytochrome b558 (genes CYBBa and CYBBb).[26,38] Approximately 65% of people have X-linked disease as a result of defects in the gp91^{phox} subunit, and the remainder have an autosomal recessive pattern of inheritance as a result of various defects in p47^{phox} (30%), p67^{phox} (< 5%) or p22^{phox} (< 5%). We did not discover disease-associated SNPs in the 3 other NADPH complex subunits. Although the NADPH oxidase components are usually considered phagocyte-specific proteins, in fact only gp91^{phox} is very phagocyte specific, whilst the other autosomal components are expressed in other tissues.[30] Thus, people who have autosomal recessive forms of CGD can also have abnormalities in tissues other than leukocytes where the affected protein is expressed, commonly vascular endothelial or renal epithelium. Hence we note with interest that several recent reports describe a juvenile nephropathy in young Boxer dogs (median age 2 yrs).[39,40] Histologic findings in these studies included tubulointerstitial inflammation, nephron atrophy, chronic pyelonephritis and focal granulomas.[40] Remarkably, *E. coli* was cultured from 88% of positive urine cultures (n=8/9).[39]

The type of *E. coli* isolated from GC-affected Boxer dogs are very similar to a new group of adherent and invasive *E. coli* (AIEC), increasingly associated with IBD in people and animal models of IBD.[20,22,41] AIEC are considered opportunistic pathogens that can exploit genetic defects in bacterial killing in an IBD susceptible individual. Patients with CGD colitis are

frequently maintained on chronic antibiotic therapy, though the specific bacteria involved are undefined, and a specific pathogenic role for *E. Coli* in CGD colitis has not yet been explored. Since the mucosally invasive bacteria are almost exclusively *E. coli* in Boxer dog GC, this implies a specific mechanism of intracellular invasion or survival involving the NADPH oxidase machinery. Elucidating the precise molecular mechanisms involved may inform knowledge of host susceptibility to microbial invasion in CGD. The overwhelming majority of infections in CGD are cited to be caused by catalase-producing organisms, predominantly *Staphylococcus aureus*, *Burkholderia cepacia, Serratia marcescens*, and *Nocardia.*[42] Systemic fungal infections, typically due to *Aspergillus* species are also common, and are the leading cause of mortality in CGD.[42] Another notable parallel here in the Boxer breed is their susceptibility to disseminated protothecal infection.[43]

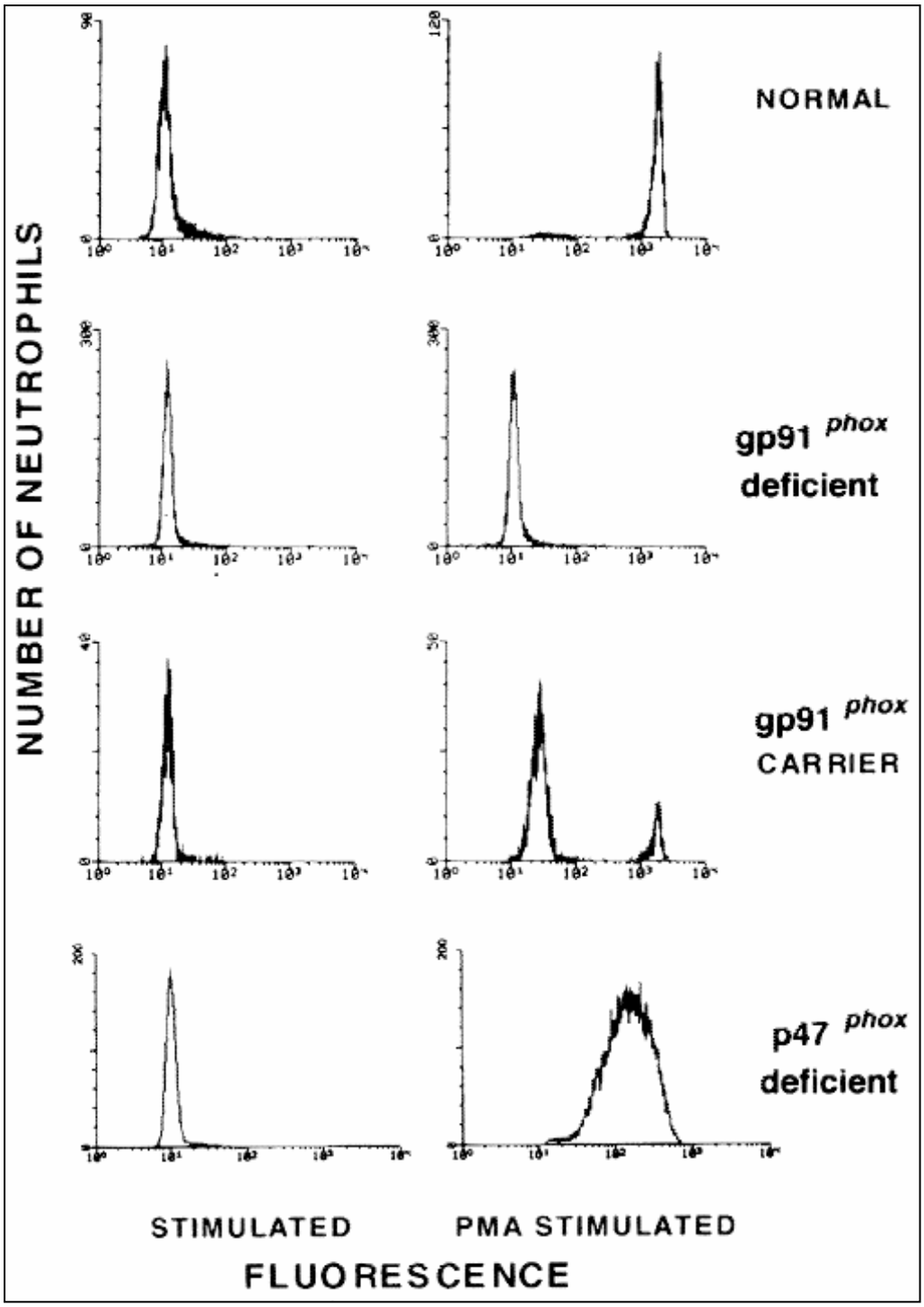
Mortality rates in GC were traditionally very high,[3,16,17,44] but the recognition of mucosally invasive *E. coli* has revolutionized disease management and prognosis. Dogs harboring an *E. coli* strain sensitive to fluoroquinolones often achieve a complete cure. On the surface this may argue against a disease phenotype with lifelong neutrophil dysfunction, however it is well recognized that the autosomal recessive forms of CGD present with a considerably milder course than X-linked forms.[26,30] In addition, a large variety of mutations are reported in CGD, such as deletions, splice site mutations and missense or nonsense mutations.[30,38,45] Depending on the type of mutation, affected people may have total absence of protein, variably decreased amount of protein, or even a dysfunctional protein product expressed at normal levels. In milder forms of CGD, such as p67^{phox} and

p47^{phox}, it is not uncommon for patients to experience one episode of disease-associated infection during their lifetime (verbal communication, Dr S.M. Holland, Laboratory of clinical infectious diseases, National Institute of Health).

The initial screening test for CGD in people is evaluation of the neutrophil respiratory burst in peripheral blood using the substrate DHR in flow cytometry.[26,46,47] We have recently evaluated a flow cytometric method of assessing the neutrophil respiratory burst in dogs and have demonstrated in two GC cases marked reductions in the neutrophil oxidative burst compared to healthy controls. Interestingly, in CGD the type of mutation gives rise to a characteristic histogram on flow cytometry (Figure 4.6), such that the affected subunit may be predicted by the shape of the histogram.[30,48] The appearance of the histograms in our 2 GC-affected dogs resembles that usually seen in human NCF2 mutations (S.M. Holland, verbal communication).

The stronger SNP associations in chromosomes 1 and 24 indicate that GC is likely to be a more complex trait than initially thought. M6P/IGF2R encodes for a multifunctional protein that binds both phosphomannosyl glycoproteins and IGF2 through independent binding sites.[49] The M6P binding site mediates lysosomal enzyme trafficking, TGF-beta activation and cell mediated cytotoxic cell death. A defect in M6P/IGF2R could clearly be of importance in Boxer dog GC, particularly with regard to lysosomal enzyme trafficking, given the intracellular survival of *E. coli* within macrophages. Polymorphisms in the M6P/IGF2R in people have been associated with variations in fetal growth, birth weight, cancer risk and Type I diabetes,[50-52] and we found no evidence of a known association with IBD. A genome wide scan in CD patients has also identified disease-associated polymorphisms in

Figure 4.6: The characteristic flow cytometry histograms associated with specific mutations in CGD, with permission, from Segal, B. H., Leto, T. L., Gallin, J. I., Malech, H. L. & Holland, S. M. Genetic, biochemical, and clinical features of chronic granulomatous disease. *Medicine (Baltimore)* **79**, 170-200 (2000). The shape of the histogram is predictive of the subunit of NADPH oxidase affected.[30]


NUMBER OF NEUTROPHILS
NORMAL
gp91 phox deficient
gp91 phox CARRIER
p47 phox deficient
STIMULATED
PMA STIMULATED
FLUORESCENCE

the organic cation transporter (OCTN). The role of these polymorphisms in CD pathogenesis is unclear, but the allelic variants form a haplotype which is reported to increase risk for ileal CD.[53]

Evidently, these findings warrant further investigation, and this can be achieved in several ways, e.g. by evaluation by a larger number of affected and control dogs, by cDNA analysis, and additional tests of neutrophil function. If a role for NCF2 in Boxer GC is confirmed, this would provide a valuable model for study of CGD and development of novel therapies e.g. new antimicrobials, gene transfer therapy. Irrespective of the long-term outcome, however, this work has served to highlight striking parallels between Boxer dog GC and human CGD that were previously unappreciated by leading experts in both fields. As a result, evaluation of a specific role for AIEC in CGD is clearly an important focus of future research. (See Appendix 1 for updated status at the time of writing).

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CHAPTER 5

Absence of a Bacterial Association in Yorkshire Terriers With Protein-Losing Enteropathy.

ABSTRACT

Yorkshire Terriers (YT) are predisposed to protein-losing enteropathy (PLE) with reported odds ratios of 4.2-10.1. Intestinal pathology of YTPLE typically includes lymphangiectasia and mucosal lymphocytic plasmacytic infiltrates. Lesions described as “dilated intestinal crypts” (JVIM 14:298-307, 2000) or “mucoid cryptal ectasia” (JAAHA 39:187-191, 2003) consisting of cystic crypts filled with mucus and necrotic cellular debris, and occasional crypt abscessation have been reported in PLE. We sought to further describe the clinical and pathological features of YTPLE and to explore a possible relationship between mucosal histopathology and mucosal bacteria.

Twelve YT with PLE were identified between 1999-2008 (7M, 5F: 3 prospective, 9 retrospective). Clinical features, intestinal biopsies and outcome were available for all 12 dogs (7 endoscopic, 5 surgical). Mucosal histopathology was examined by a blinded pathologist and inflammatory infiltrates, lymphangiectasia and crypt abnormalities were scored as normal-0, mild-1, moderate-2 or severe-3. Fluorescence in situ hybridization (FISH) with a eubacterial probe was used to ascertain the presence and distribution of bacteria in duodenal biopsies.

The median age and bodyweight at presentation were 92mo, and 3.1kg, respectively. Vomiting (7), diarrhea (6) and inappetance (5) were the

most frequent clinical signs. Bicavity effusions were present in 5 dogs, and ascites alone in 3. Hypoalbuminemia (< 3.1g/dl) was present in all 12 dogs (median 1.6g/dl), and hypoglobulinemia (<1.9g/dl) in 7 (median 1.7g/dl). Additional biochemical abnormalities included hypocalcemia (12), hypocholesterolemia (11) hypomagnesemia (9), hypokalemia (5) and hypochloremia (5). Hematological abnormalities included mild anemia (5), thrombocytosis (8), mature neutrophilia (6), and neutrophilia with a left shift (n=3). Anti-thrombin III was low (<75%) in 4/6 dogs evaluated (mean 62%). Duodenal biopsies from all affected YT contained cystic intestinal crypts. Lymphangiectasia (median 2, range 0-3), crypt hyperplasia (median 2, range 1-3) and villus blunting (4 dogs), were less consistent features. Mucosal infiltration of lymphocytes and plasma cells (villus median 2, range 1-3; crypt median 3, range 2-3) and eosinophils (median 1.5, range 1-2) was common. Empirical therapy with corticosteroids (11 of 12), azathioprine (2/12), antibiotics, plasma and diuretics was associated with a poor outcome. 7 of 12 cases died or were euthanased within 3m of diagnosis. Long-term survival occurred in 3 dogs, (36, 24, and 8m), and 2 are alive at 3m and 4m after diagnosis. FISH analysis showed no evidence of a bacterial association with crypt cysts or with mucosal inflammation.

We conclude that YT suffer a severe and often fatal form of PLE that is consistently associated with cystic intestinal crypts. The absence of a bacterial association suggests that this may be a primary morphogenetic disorder with or without a secondary environmental trigger. Further work is required to ascertain the etiopathogenesis of crypt lesions and their relationship to enteric protein loss.

INTRODUCTION

Protein losing enteropathy (PLE) is the term applied to intestinal disorders that cause leakage of protein into the intestinal lumen. Breeds known to be predisposed to PLE include the Soft Coated Wheaten Terrier (SCWT), Basenji, Lundehund, Shar Pei, Rottweiler and Yorkshire Terrier.[1-8] PLE is reported to be associated with a variety of intestinal diseases, including inflammatory bowel disease (IBD),[9,10] lymphangiectasia,[6,11-13] immunoproliferative enteropathy,[14] histoplasmosis,[15, 16] and neoplasia.[17] In adult dogs, PLE is most often caused by idiopathic IBD, lymphangiectasia, or lymphoma, most likely involving pathophysiological mechanisms such as altered immune function, environmental factors (e.g. diet), microbial and genetic factors.[18-22] There are very few studies in dogs exploring specific molecular mechanisms of intestinal protein loss, but from a biologic standpoint this could include hemorrhage, ulceration, erosion, lymphatic damage and leakage from intercellular junctions.[7] Reports of SCWT PLE suggest that the primary event is disruption of lymphatic structures, leading to lymphatic blockage, dilation, and protein leakage.[22] Alternatively the principal lesion could be inflammatory, with lymphatic dilation and leakage developing secondary to lymphangitis and lipogranulomas.[5] Evaluation of intestinal biopsies from Lundehund PLE by electron microscopy revealed rupture of lacteals, suggesting enhanced hydrostatic tissue pressure and increased paracellular permeability.[23]

Recently, intestinal crypt lesions characterized by numerous crypt cysts have been associated with PLE but is unclear however whether crypt pathology is the actual mechanism for protein loss, or merely a nonspecific

marker of disease.[4] The Yorkshire terrier is over-represented for PLE in the US as well as Europe, with an odds ratio of 10.1 in one hospital population.[8,22,24] Hospital records at Cornell University Hospital for Animals (CUHA) show that Yorkshire Terriers with PLE (YTPLE) are particularly susceptible to crypt pathology. Crypt cysts are often described as 'necrosis' or 'abcessation' by pathologists, although it remains to be seen whether or not they are associated with an infectious etiology. In a previous report, it was observed that parvovirus infection results in a similar histological appearance to YTPLE, i.e. villus collapse and fusion, and dilated protein/mucus-filled crypts, but immunostaining for parvovirus antigen in 2 dogs was negative.[6]

Crypt abscesses and cryptitis are early lesions observed in the main forms of IBD in people, Crohn's disease (CD) and ulcerative colitis (UC).[24] Crypt lesions consisting of dilated crypts containing polymorphonuclear cells were found to be highly predictive of colonic IBD, and particularly UC, as opposed to CD.[25,26] Increased numbers of mucosa-associated bacteria have been found to colonize colonic crypts relative to healthy human controls.[25] Taken together, these findings suggest a bacterial association in crypt abscessation, but surprisingly the single study that specifically looks for bacteria in crypt abscesses did not find a convincing bacterial association.[27] Nevertheless, the importance of infectious agents is increasingly recognized in the pathogenesis of canine and human IBD,[25] and an excellent example of a specific pathogen in canine IBD is *E. coli*-associated granulomatous colitis (GC) of the Boxer dog.[28, 29] Eradication of mucosally invasive *E. coli* in GC has transformed treatment and prognosis, highlighting the potential for, and importance of identifying breed-specific bacterial susceptibilities.[30]

The aim of this work was to describe the clinical characteristics, treatment and outcome of YTPLE. Our second aim was to determine the incidence of crypt lesions in YTPLE, and to explore the relationship of bacterial infection to crypt 'cysts/abscesses.'

METHODS

Animals

Clinical and pathology databases were used to retrospectively identify Yorkshire Terriers referred to CUHA for investigation of gastrointestinal (GI) disease. Search parameters included YT which underwent small intestinal biopsy, hypoproteinemia, hypoalbuminemia, and ascites. Cases identified prospectively during the study duration were also included. Historical and clinical parameters, and long-term outcome were obtained from CUHA and rDVM case files, and by direct follow-up in prospective cases.

Intestinal histology

Intestinal biopsies were blindly evaluated by a board-certified veterinary pathologist. Inflammatory infiltrates, lymphangiectasia and crypt abnormalities were scored as normal-0, mild-1, moderate-2 or severe-3.

FISH analysis

Formalin-fixed, paraffin-embedded archival intestinal biopsies were recut to 4 micron thickness and mounted on Probe-On Plus slides (Fisher Scientific, Pittsburgh, PA) for screening by fluorescence in situ hybridisation

(FISH) with a eubacterial 16S rDNA probe (EUB-338, GCTGCCTCCCGTAGGAGT). Slides were deparaffinized by passage through xylene, 100% alcohol, 95% ethanol, and finally 70% ethanol (20 min) and air-dried. FISH probes 5' labeled with Cy3 or 6-FAM (Integrated DNA Technologies, Coralville, IA) were reconstituted with sterile water to a working concentration of 5 ng μl^{-1} with hybridization buffer (20 mM Tris-HCl, 0.1% sodium dodecyl sulfate [SDS], 0.9% NaCl [pH 7.2]). Hybridization with 30 µl of DNA probe mix in a hybridization chamber occurred at 46°C overnight. Slides were washed in hybridization buffer minus SDS at 48°C for 30 min, before final washing in phosphate-buffered saline (PBS), and mounted when dry with a ProLong antifade kit (Molecular Probes Inc., Eugene, OR). Sections were examined on an Axioskop 2 (Carl Zeiss Inc., Thornwood, NY) or a BX51 (Olympus America, Melville, NY) epifluorescence microscope, and images were captured with a Zeiss Axiocam or Olympus DP-7 camera, respectively. Slides spotted with *E. coli*, *Staphylococcus* spp. and *Streptococcus* spp. were used as positive controls. Probe specificity was additionally evaluated by counter-staining with the irrelevant probe non-EUB-338 (ACTCCTACGGGAGGCAGC).

RESULTS

Clinical parameters

Twelve YT diagnosed with PLE were identified from hospital records between 1999-2008 (7M, 5F: 3 prospective, 9 retrospective), with median age 92 months and bodyweight 3.1 kg. The most frequent clinical signs (Table 5.1)

were vomiting, inappetance, and diarrhea, and the median duration of clinical signs prior to diagnosis was 6 weeks (range 1-7 weeks)

Table 5.1: Predominant clinical signs in 12 YT with PLE

CLINICAL SIGN	# OF DOGS (%) (n=12)
Diarrhea	58
Inappetance	50
Vomiting	50
Bicavity effusion	42
Lethargy	33
Ascites	25
Weight loss	17
Anorexia	8
Dyspnea	8
Melena	8
No GI signs	8

Laboratory parameters

The predominant clinicopathological abnormalities (Table 5.2) were panhypoproteinemia, hypocalcemia, hypo-magnesemia, and hypocholesterolemia. Folate and cobalamin, measured in 5/12 dogs, were within normal limits. Faecal analysis was negative for enteric parasites, and culture was negative for enteric pathogens such as *Salmonella* and *Campylobacter*.

Table 5.2: Biochemical and hematological abnormalities in 12 YT with PLE.

Biochemical parameter	**#/12**	**Median (ref. range)**	**Hematological parameter**	**#/12**	**Median (ref. range)**
Hypoalbuminemia	12	1.6 g/dL (3.1-4.1)	Anemia	5	36% (42-57)
Hyoglobulinemia	7	1.65 g/dL (3.9-5.3)	Neutrophilia	9	15.25 thou/uL (3.4-9.7)
Hypokalemia	5	3.6 mEq/L (3.8-5.3)	Left shift	3	0.9 thou/uL (0-0.1)
Hyperchloremia	5	128 mEq/L (109-117)	Thrombocytosis	8	790 thou/uL (179-483)
Hypomagnesemia	9	1.0 mEq/L (1.4-2)	Low anti-thrombin III	4/6	63.5% (75-120)
Hypocholesterolemia	11	94 mg/dL (124-335)	**Other**		
Hypocalcemia	12	6.5 mg/dL (9.3-11.6)	Hyperfibrinogenemia	3	743 mg/dL (147-479)
Hypophosphatemia	1	2.3 mg/dL (3.3-6.0)	Isosthenuria	4	1.012
Hyperamylasemia	8	1591.5 U/L (286-1124)	Hyposthenuria	2	1.004

Intestinal pathology

Endoscopic duodenal biopsies were evaluated in 7 dogs, and full thickness surgical biopsies in 5. Typical histological findings included lymphoplasmacytic inflammation, usually more severe in the crypts than the villi, eosinophilic infiltrates, and lymphangiectasia (Table 5.3, Figure 5.1 a-d). Crypts were frequently severely dilated and appeared to contain debris, mucus, macrophages and neutrophils. They were lined by enterocytes which appeared hyperplastic in some cases. Frequently, crypts were so large that they had ruptured to the surface, spilling their proteinaceous content into the intestinal lumen. Notably, severe hypoalbuminemia was present in 3 dogs with no, or only mild lymphangiectasia.

Table 5.3: Histological findings in 12 YT with PLE.

Dog	Lymphoplasmacytic inflammation VILLUS	CRYPT	Eosinophils	Lymphangiectasia	Crypt cysts
1	2	3	1	2	2
2*	2	3	2	2	1
3	3	3	1	2	3
4	2	3	1	2	3
5*	2	3	2	2	1
6	2	3	1	3	3
7	2	3	2	1	2
8*	1	3	2	3	3
9	2	3	2	3	3
10	1	3	1	1	2
11	1	3	1	0	1
12	2	3	2	1	3
MEDIAN	**2**	**3**	**1.5**	**2**	**2.5**

normal-0, mild-1, moderate-2, severe-3.

*villus blunting present

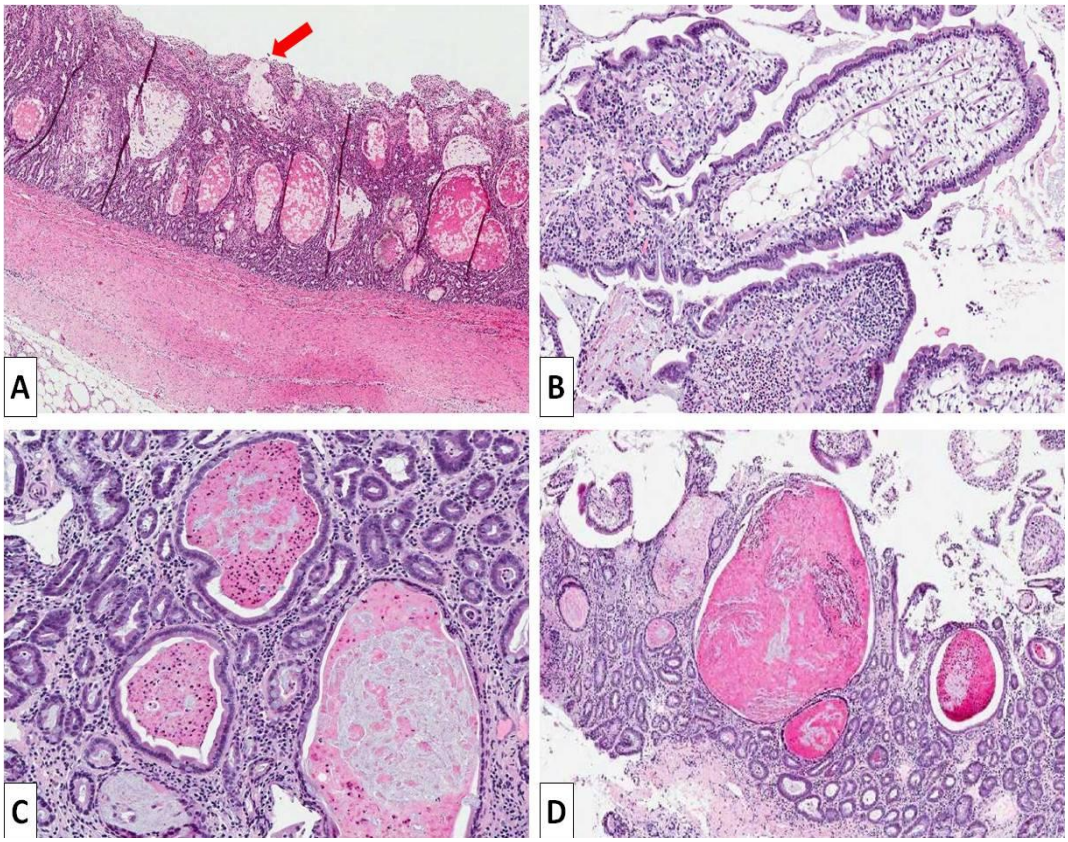

Figure 5.1: **(A)** YTPLE full thickness small intestinal biopsy showing numerous very large crypt cysts in the mucosa and submucosa, and rupturing at the surface (arrow) (H&E, Aperio digital scan, 2X) **(B)** Lacteal dilation and severe LP inflammation (H&E, Aperio digital scan, 8X) **(C,D)** Small intestinal crypt cysts filled with proteinaceous debris and sloughed cells (H&E, Aperio digital scan, x10)

Eubacterial FISH

FISH analysis using a eubacterial probe to target 16S rDNA revealed very few mucosa-associated bacteria in all 12 dogs. We found no evidence of bacteria within crypt cysts or in lipogranulomas (Figure 5.2).

Treatment and outcome

The majority of dogs received immunosuppressive treatment with either corticosteroids (prednisolone, 0.7-1.8 mg/kg/day) or azathioprine, and many also received antibiotics (metronidazole, enrofloxacin, amoxillin-clavulanate, cephalexin, tylosin, ampicillin, doxycycline). Dietary change was attempted in a number of cases, but was very difficult to implement due to poor appetite. Treatments administered are summarized in Table 5.4.

Table 5.4: Summary of treatments administered to YTPLE cases

TREATMENT	**#/12**
Prednisolone	9
Dexamethasone	2
Azathioprine	2
Antibiotics	9
Diuretics	1
Plasma	1
Hetastarch	4
Human albumin	1
Gut protectants	8
Ultralow dose aspirin	5
Cyclosporine	3

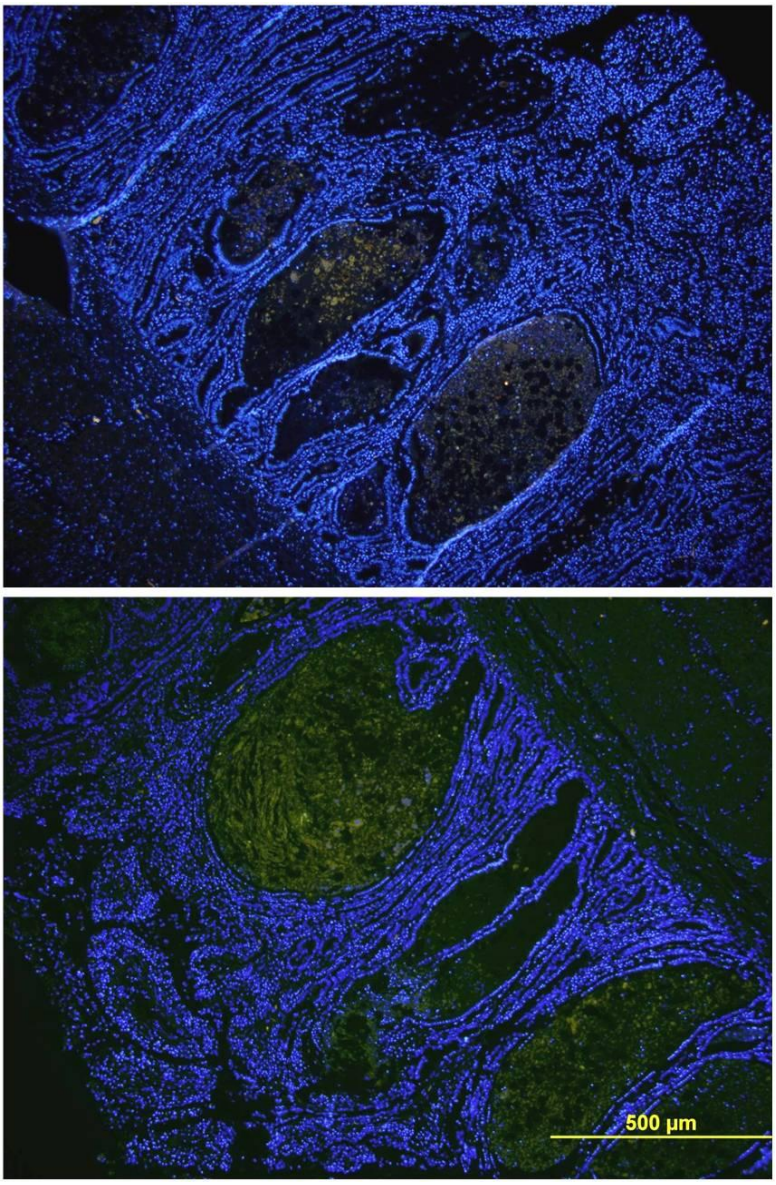

Figure 5.2: FISH showing large crypt ‘cysts’ in a duodenal biopsy from YTPLE. 16S rDNA eubacterial probe EUB338-Cy3 (red), non-EUB-6FAM (green), DAPI (blue) stains cell nuclei (x40).

Treatment was generally met with poor success, and 58%, (7/12) died, or were euthanased within 3 months of diagnosis. Three of these cases died very suddenly at home despite 2/3 appearing to respond to treatment, and we speculate that this was a consequence of thromboembolism. Longer-term survival occurred in 3 dogs (10, 24, and 36 months) before death or euthanasia due to disease. Two dogs are alive at 24, and 26 months after diagnosis; one remained hypoalbuminaemic, with albumin levels ranging from 1.4-1.9 mg/dL (3.1-4.1), whereas serum proteins normalized in the other. The treatment received by these 2 cases included dexamethasone (0.1 mg/kg/day subcutaneously), cyclosporine (5mg/kg every 12 hrs) and ultralow dose aspirin (approx. 0.5mg/kg/day).

DISCUSSION

We have shown here that the YT breed is susceptible to PLE with a unique phenotype, characterized by development of large, multifocal crypt cysts or abscesses. FISH analysis revealed no evidence of bacterial DNA, hence they appear to be sterile structures. Protein loss associated with crypt pathology is particularly severe, and poorly responsive to the usual management strategies advocated for other forms of canine IBD, i.e. dietary change, antimicrobials and immunosuppression. High mortality rates occur, and even in apparently responding dogs there is risk of sudden death due to suspected thromboembolism. Although the mechanisms underlying their development are unknown, we postulate that crypt cysts/abscesses are an important mechanism of enteric protein loss in YTPLE, if not the primary lesion.

There exist only 2 previous reports of crypt lesions associated with PLE, in a total of 8 dogs, and 2/8 were Yorkshire Terriers.[31,32] The material contained within crypt cysts/abscesses remains to be elucidated, but their appearance on H&E stained sections and with DAPI (which stains cell nuclei) is consistent with sloughed cells, denuded cell nuclei, and mucus/debris. Thus, perhaps 'sterile abscess' is a more apt descriptor than 'cyst.' The precise manner in which protein loss occurs in YTPLE is unclear, but if the sheer number of crypt abscesses in the sections examined is representative of a large surface area of small intestine, this would likely result in significant protein wasting. These massively distended structures appear to rupture at the mucosal surface, and if the cells lining the abscess possess secretory functions, severe ongoing protein loss could ensue. Previous authors have suggested that severe crypt dilation compresses the base of the adjacent villi, leading to lymphatic obstruction.[8,24]

In other breeds, such as the SCWT, the principal mechanism of protein loss is thought to occur via disruption of lymphatic structures, leading to lymphatic dilation and blockage.[13] Also suggested is the possibility that the principal lesion may be inflammatory, and lymphatic dilation occurs secondary to loss of serum proteins into the alimentary tract.[5] Basenji enteropathy, another breed-specific PLE, is referred to as an immunoproliferative process due to intense mucosal infiltration with lymphocytes and plasma cells, and protein loss in this disease is thought to be associated with damaged intercellular junctions as well as lymphangiectasia.[5] Evaluation of intestinal biopsies from Lundehund PLE by electron microscopy revealed rupture of lacteals, suggesting enhanced hydrostatic tissue pressure and increased

paracellular permeability.[14] In contrast, in YTPLE, intestinal pathology is unique in that the frequency and size of crypt cysts far outweighs the presence and severity of lymphatic abnormality, which was minimal in many cases.

From a comparative aspect, diseases occurring in people that share some characteristics of YTPLE are Ménétrier disease, Chronkite-Canada syndrome (CCS), and tufting enteropathy.[33-40] Menetrier disease (MD) is a rare and severe protein losing gastroenteropathy, also known as hyperplastic hypersecretory gastropathy, whereby gastric epithelial hyperplasia results in formation of glandular and mucosal cysts.[33,34] Interestingly, MD has been associated with cytomegalovirus in children, and *H. pylori* infection in adults and treatment of these infections can reverse the disease process.[35,36] CCS is a very rare and poorly understood PLE characterized by marked epithelial proliferation, polyposis, and diffuse dilation of intestinal glands which release protein-enriched secretions into the gut lumen.[37] The disease with perhaps the most similar crypt lesions to YTPLE seen in people is ‘tufting enteropathy’, a form of epithelial dysplasia characterized by partial villous atrophy, crypt hyperplasia, pseudocystic crypt appearance, and disorganization of surface epithelium.[38] This disease was recently shown to be caused by mutations in the gene encoding epithelial cell adhesion molecule (EpCAM),[39,40] which points to the need to evaluate intercellular adhesion molecules in YTPLE.[41]

We did not discover any evidence of bacterial involvement on FISH analysis, but one caveat of this is that in order to take up the fluorescent probe, bacteria must be metabolically active. Dead bacteria, or failure of the FISH probe to penetrate the abscess wall cannot be excluded, but this seems somewhat unlikely since in general we visualized extremely scant mucosa-

associated and intraluminal bacteria. A role for other infectious agents, such as fungal and viral organisms has not been excluded. Viral organisms that can induce similar intestinal pathology to YTPLE are parvovirus and coronavirus, which cause villus fusion, and dilated mucus filled crypts lined by hypertrophic enterocytes. In a previous report, immunohistochemical staining of crypt cysts for parvovirus antigen was negative, but this included only two cases and a larger number should be evaluated before drawing firm conclusions.[39]

Ulcerative colitis (UC) and Crohn's disease (CD) are the 2 most common types of IBD in people, and although their pathophysiology is still unknown, the gut microbiota is considered to play a crucial role.[24] Crypt abscesses are reported to be early lesions of UC in particular, and they are very similar in appearance to YTPLE, containing cell debris, mucus and degenerating neutrophils.[28,42] A recent study theorized that crypt abscesses in human IBD develop as a result of localized dysbiosis arising from bacteria colonizing the crypts.[25,43] However, in line with our results, this was not supported by FISH evaluation of crypt abscesses in either CD and UC,[25] which revealed colonization of crypts in 42.9% of people with acute colitis and only 3.6% of IBD patients, versus 0% of healthy controls. The authors concluded that crypt lesions in UC and CD are aseptic abscesses, involving pathogenesis other than dysbiosis.[25]

Increased risk of hypomagnesemia and hypocalcemia has been previously reported in YTPLE, and were observed here in 75% and 100% of cases respectively. Mechanisms for hypomagnesemia are suggested to include gastrointestinal losses due to diarrhea and malabsorption, and increased G.I. permeability.[25] Clinical manifestations of hypomagnesemia, for

example weakness, tremor, hyporeflexia, dysrhythmias were not appreciated here. Calcium malabsorption could be associated with vitamin D deficiency; hypomagnesemia can also contribute hypocalcemia by impaired function of parathyroid glands and reduced responsiveness to PTH[6]. Interestingly, a left shift, hyperfibrinogenemia and thrombocytosis were not uncommon findings, reflecting a systemic inflammatory response. It is possible that these may be useful markers for YTPLE, but since they are not specific to the GI tract, their evaluation in other inflammatory diseases in the YT is warranted.

Evidence of inflammation in YTPLE supports the use of anti-inflammatory agents in treating YTPLE, but responses to treatment with corticosteroids and azathioprine were variable, and no consistent benefit was seen in clinical parameters or serum proteins. Given our limited understanding of pathogenesis, treatment was empirical with diet, antibiotics, and immunosuppression. In our experience responses to treatment in YTPLE are very short-lived, and minimal improvement in albumin levels is normally seen. In the two surviving dogs a different protocol was used compared to earlier cases, to include cyclosporine and dexamethasone (SQ) with ultra-low-dose aspirin. These are too few cases to determine whether or not the longer survival is really treatment-related, but this approach deserves further evaluation. Serum cyclosporine levels did not reach recommended therapeutic levels in either case, but it is possible that a topical effect may be occurring. Inflammation with an eosinophilic component was a common histologic finding, perhaps suggesting immunoreactivity to luminal antigens e.g. diet or microbes. Implementing dietary change in these cases was extremely difficult due to poor appetite. Affected YT are often muscle and ascitic at presentation,

and inability to increase dietary protein intake makes management extremely difficult. There is an argument for aggressive nutritional support from the offset, with placement of an esophagostomy or gastrostomy tube at the time of intestinal biopsy, as deemed appropriate depending on the presence of vomiting and severity of ascites.

In people, PLE develops as a symptom in ostensibly unrelated diseases, including CD, congenital disorders of glycosylation, systemic lupus erythematosus, and as a complication of Fontan surgery to correct congenital heart malformations.[6] A recently noted commonality of PLE-associated disease in people is the loss of heparan sulfate (HS) and the heparan sulfate proteoglycan (HSPG) syndecan-1 (Sdc1), from the basolateral surface of intestinal epithelial cells during PLE episodes. When PLE resolves, HS and Sdc1 have been shown to reappear, which implies their involvement in PLE pathogenesis.[44] This is further supported by research in mice with either Sdc1 gene deletion or intestinal-specific induced loss of HS that were far more susceptible to protein loss, particularly when also exposed to combinations of pro-inflammatory cytokines IFN-γ, TNF-α. Evidence from a clinical viewpoint is that both high and low-molecular-weight heparin (LMWH) reverse PLE in people post-Fontan surgery,[45] suggesting a rationale for heparin therapy in PLE patients. Clinical trials to assess non-anticoagulant HS in human PLE are currently underway, and this may in future prove to be a useful aspect of management in dogs. Therapy with unfractionated, or LMWH could obviously have dual benefit in YTPLE due to their apparent propensity for development of thromboembolism, and this warrants further management.

In summary, YT suffer a severe, and often fatal form of PLE that is consistently associated with sterile abscessation of intestinal crypts. Further work is required to ascertain the etiopathogenesis of crypt lesions and their relationship to enteric protein loss. The discovery of EpCAM mutations in human PLE, and the YT breed predisposition to this unique pathology suggest a genetic basis, for which newly developed tools e.g. genome wide association study may facilitate identification of candidate genes.

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CHAPTER 6

High Throughput Pyrosequencing Reveals Dysbiosis in Canine IBD.

ABSTRACT

The enteric microflora is increasingly implicated in the etiopathogenesis of inflammatory bowel disease (IBD). Recent work has revealed reduced bacterial diversity and a shift towards a Gram negative flora in the inflamed intestine across species, but the relationship of individual components of the enteric flora to different disease syndromes and clinical response is unclear. We sought to determine if the duodenal mucosal flora of dogs with IBD differs from healthy dogs, and to gain insight into the relationship of the mucosal flora to clinical response to diet, antibiotics, or immunosuppression.

A culture-independent bacterial tag-encoded FLX 16S rDNA amplicon pyrosequencing (bTEFAP) approach was used to evaluate the microbiome of endoscopic duodenal biopsies from 16 dogs with a histological diagnosis of IBD (9F, 7M, median age 9.9 yrs) and 14 clinically healthy (H) dogs (8F, 6M, median age 7.5 yrs). 10 dogs were food responsive (FR), 3 steroid responsive (SR), and 3 were partially responsive to a combination of food and antibiotics (FAR). A total of 85,065 raw sequences (54776 from H, 30289 from IBD) were analyzed. The number of sequences per individual (median) was higher in H (3,912) than IBD (1,893) ($p=0.007$). Dogs with IBD had lower overall species diversity than H at all primary levels of taxonomy based upon rarefaction ($p<0.05$). When evaluated at Order level, we observed significant differences in the proportions of sequences in 16S rDNA libraries from H vs. IBD

(***p<0.001, **p<0.01. *p<0.05) due to enrichment in IBD of *Actinomycetales*,*** *Bacteroidales*,** *Burkholderiales*,*** *Lactobacillales*,** *Erysipelotrichales*,* *Rhizobiales,** and *Xanthomonadales*.* and depletion of *Bacteroidales*.* Analysis by clinical response indicated differences in numbers of sequence between groups (H, FR, SR and SA: Kruskal Wallis, P<0.003,), and lower numbers in FR versus H (p=0.007). Order-level comparison revealed greater relative proportions in FR compared to H of: *Actinomycetales*,*** *Bacteroidales*,*** *Burkholderiales*,** *Erysipelotrichales*,* *Lactobacillales*, *Rhizobiales** and *Xanthomonadales*,* and depletion of *Bacteroidales*.

We conclude that duodenal inflammation is associated with decreased diversity of the mucosal flora, and altered relative proportions of dominant floral groups. Further work is required to determine whether differences in the mucosal flora impact the clinical response.

INTRODUCTION

Idiopathic inflammatory bowel disease (IBD) is the most common cause of chronic gastrointestinal (GI) signs in dogs.[1-3] Clinical signs vary depending on the region of the GI tract affected, but diarrhea, vomiting, inappetance and weight loss occur most frequently.[4,5] In dogs, IBD is classified according to histological characteristics, i.e. lymphocytic-plasmacytic, eosinophilic, granulomatous. The most prevalent forms of human IBD are Crohn's disease (CD) and ulcerative colitis (UC), which are distinctly different to canine IBD in terms of histology (mucosal versus transmural in CD), complications (e.g.

intramural abscessation, intestinal obstruction, perforation), and extraintestinal manifestations (e.g. arthralgias, hepatobiliary disease).[6,7] However, commonalities exist in the mechanisms responsible for development of IBD across species, i.e. environmental factors, genetic factors, immunoregulatory defects, and luminal microbes.[2,6,8,9] The specific mechanistic and temporal contributions of each individual factor, and their complex interrelationships are poorly understood.[8] Consequently treatment of IBD, especially in dogs, is largely empirical, using combinations of dietary manipulation, antimicrobials and anti-inflammatory and immunosuppressive medications.[2,4,5] Responses to treatment are variable and highly individual, and are not governed by the site, the type, or the severity of disease.[4,5] Currently, we have no objective, non-invasive biomarkers of disease, or reliable predictors of response to guide treatment. Not infrequently therefore, management can involve a lengthy period of trial and error, which can be a costly and frustrating process, particularly in poor responders, resulting in euthanasia in 13-18% of cases.[4,5]

It is increasingly recognized that a dynamic imbalance between resident microbes and host defenses plays a pivotal role in the initiation and maintenance phases of IBD.[9-13] The evidence arises from a variety of disciplines including genetics, molecular microbiology, rodent models, translational research, and clinical trials.[8,10,14,15] In strong support of a role for the microflora are studies in gnotobiotic animal models, showing that enteric bacteria are crucial for development of chronic inflammation,[16] and experiments in germ-free animals revealing a bacterial specificity for inducing inflammation (*Bacteroides*, *E. coli, Enterococcus*).[17] Clinical evidence is abundant in CD patients, e.g. positive responses to antimicrobials and clinical

improvements due to fecal stream diversion that deteriorate when the bypassed ileum is reperfused with ileal contents,[18,19] Studies utilizing fluorescence in situ hybridization (FISH) with probes directed against bacterial 16S rDNA have shown that the mucosa-associated flora is increased in UC and CD, with enrichment of *Proteobacteria*, *Enterobacteriaceae* and *Bacteroides* predominating in most studies.[12,20,21]

The genetic variants that confer risk of CD also highlight the importance of innate immunity (i.e. NOD2 gene), autophagy (ATG16L, IRGM genes) and phagocytosis (NCF4 gene) in disease pathogenesis.[15,22,23] NOD2 encodes an intracellular pathogen recognition receptor (PRR) which recognizes bacterial muramyl dipeptide and initiates the NF-kB pathway to activate host defenses.[24] People with NOD2 polymorphisms have an increased propensity to development of Crohn's disease, for reasons which are not fully understood but involve defective bacterial sensing and diminished production of antibacterial defensins.[25] Recent work on German Shepherd dogs with IBD also identified polymorphisms in the NOD2 gene, along with other PRR genes, TLR 2, 4 and 5. The autophagy pathway genes ATG16L and IRGM, are important in clearing intracellular microbes and apoptosis, and have been implicated in CD pathogenesis by genome wide association study (GWAS). A recent GWAS of CD has also revealed polymorphisms in NCF4,[26] a component of the phagosomal enzyme NADPH oxidase that is required for generation of the phagocyte respiratory burst and bacterial killing.[27] Emerging work in Boxer dog granulomatous colitis suggests that polymorphisms in another NADPH oxidase component, NCF2, may confer risk of intramucosal

bacterial invasion. Clearly the common theme emerging in IBD genetics is abnormal interfacing of host defenses with the resident enteric microflora.

The canine intestinal microbiome is relatively poorly defined in health and disease, but a role for luminal bacteria is implied by the clinical response to antibiotics such as metronidazole and tylosin.[28-30] Due to significant advances in molecular microbiology, including 16S rDNA clone library construction and 454-pyrosequencing, recent studies have shown enrichment of the *Enterobacteriaceae* in dogs with duodenal IBD, and decreased microbial diversity.[31-33] However, so far these studies have been purely descriptive and make no attempt to examine the relationship of shifts in the microbiome to other phenotypic parameters or outcome.

Our aim here was to test the hypothesis that dogs with IBD have an abnormal mucosa associated flora that directly impacts mucosal and systemic inflammation and clinical outcome. By undertaking this in-depth disease profiling, we hope to contribute to the establishment of an evidence-based approach in the management of canine IBD.

METHODS

Animals and clinical phenotypes

- *Diagnosis*

Adult dogs of any age or breed presented to Cornell University Hospital for Animals for the investigation of G.I. disease were prospectively evaluated. Inclusion criteria included a history of chronic GI signs i.e. one or more of

vomiting, diarrhea, weight loss, anorexia, for > 1 month. Routine investigation of clinical signs was performed as dictated by individual case need and recent rDVM diagnostics. The minimum database included: CBC, serum biochemistry fecal flotation and culture, *Giardia/Cryptosporidium* ELISA, serum TLI, folate, B12, +/- spec cPL, urinalysis, and abdominal ultrasound. No unnecessary or additional tests were performed for study purposes, except for C-reactive protein, which was assayed as an indication of systemic inflammatory response (Tridelta commercial canine ELISA). Owner consent was granted to obtain 8-10 additional mucosal biopsies during upper GI endoscopy.

Dogs were included only if histological evaluation of endoscopic biopsies by an ACVP boarded pathologist was consistent with IBD, and they had received no medication during at least the previous two weeks. In some instances, in dogs receiving antibiotics, endoscopic biopsy was delayed to meet the 2-week washout period provided the animal was stable. The study was approved by Cornell University Institutional Animal Care and Use Committee (IACUC# 2007-101 and 2007-0014).

- *Treatment (Figures 6.1 and 6.2)*

A standardized approach to treatment was adopted in all normoproteinemic cases, consisting of:

1. Dietary change to a commercial hydrolyzed soy protein (Purina HA®), fed according to individual requirements and the manufacturers' recommendations. Fenbendazole was also administered to better exclude enteric parasites such as *Giardia* (50mg/kg per os for 5 days).

2. After 14 days, responsive cases remained on Purina HA®. Non-responsive cases received Tylosin, 15mg/kg every 12 hrs per os (and remained on Purina HA, provided clinical signs had not worsened).

3. After 14 days, responsive cases remained on HA® and tylosin. Dogs not responsive to (1) or (2) remained on these treatments provided clinical signs had not worsened, and also received prednisolone, 2mg/kg/day (in dogs <70lbs). In dogs >70lbs, azathioprine was started instead, at 2mg/kg/day for 5 days, then on alternate days (in order to avoid an unacceptable level of steroid-associated adverse effects in large breed dogs).

4. After 14 days, animals not responsive to (1), (2), or (3) also received azathioprine, (2mg/kg/day for 5 days, then on alternate days). Those on azathioprine already also received prednisolone, 1mg/kg/day.

In 2 independent studies, hypoalbuminemia has been identified as a negative prognostic indicator in canine IBD,[4,5] which suggests a need for more aggressive management. Thus, dogs with albumin <2.0 g/dL, received treatment with prednisolone from the offset, (1mg/kg every 12 hrs) alongside dietary change to Purina HA®. In dogs that showed no improvement after two weeks, and in dogs >70lbs (in order to avoid an unacceptable level of steroid-associated adverse effects), azathioprine was administered (2mg/kg/day for 5 days, then on alternate days). In both treatment protocols, completely non-responding cases were thoroughly re-evaluated to ensure correct diagnosis, and repeat endoscopic biopsy for a more objective measure of response was considered.

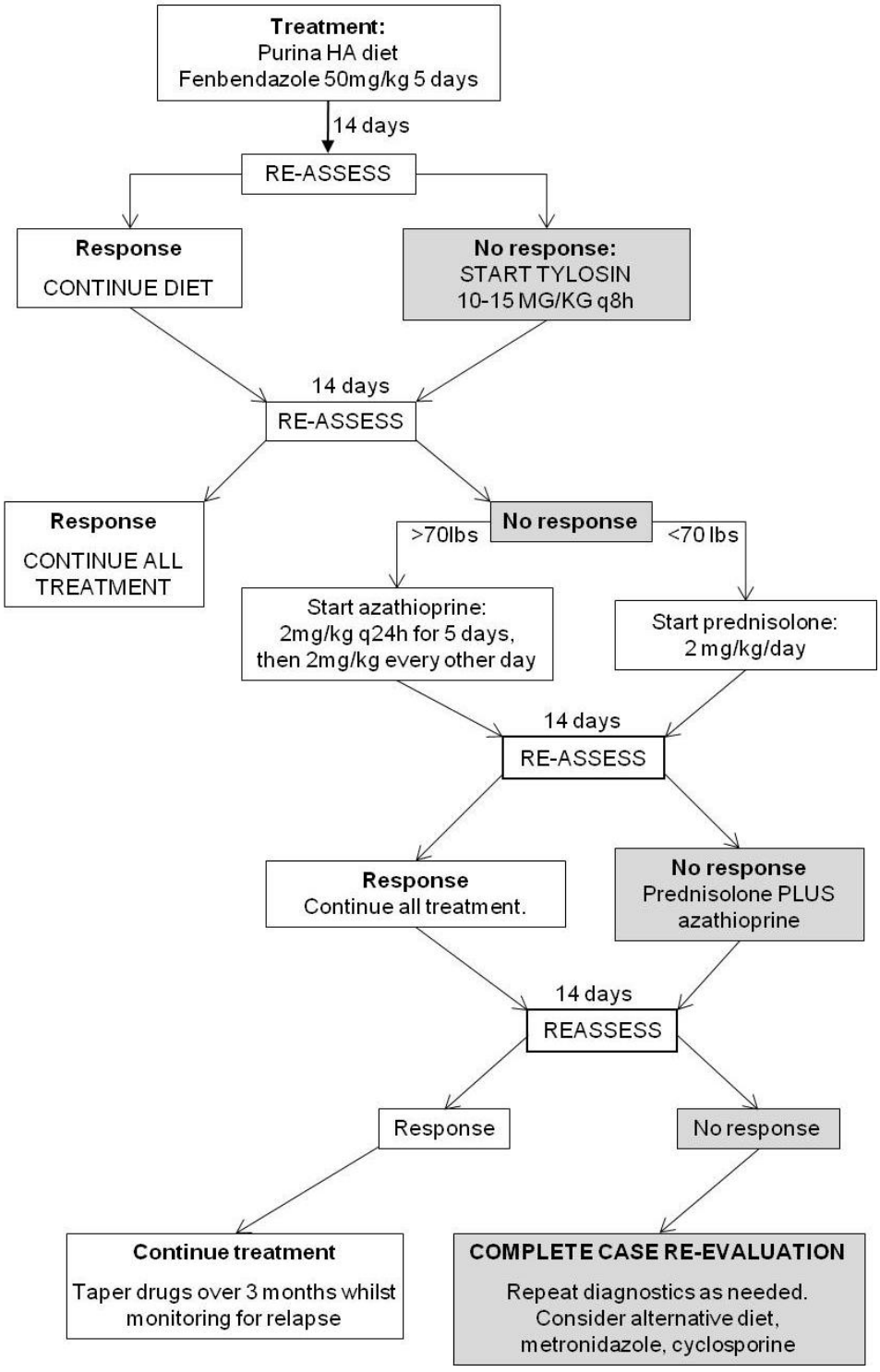


Figure 6.1: Treatment flowchart for dogs with albumin >2.0g/dl.

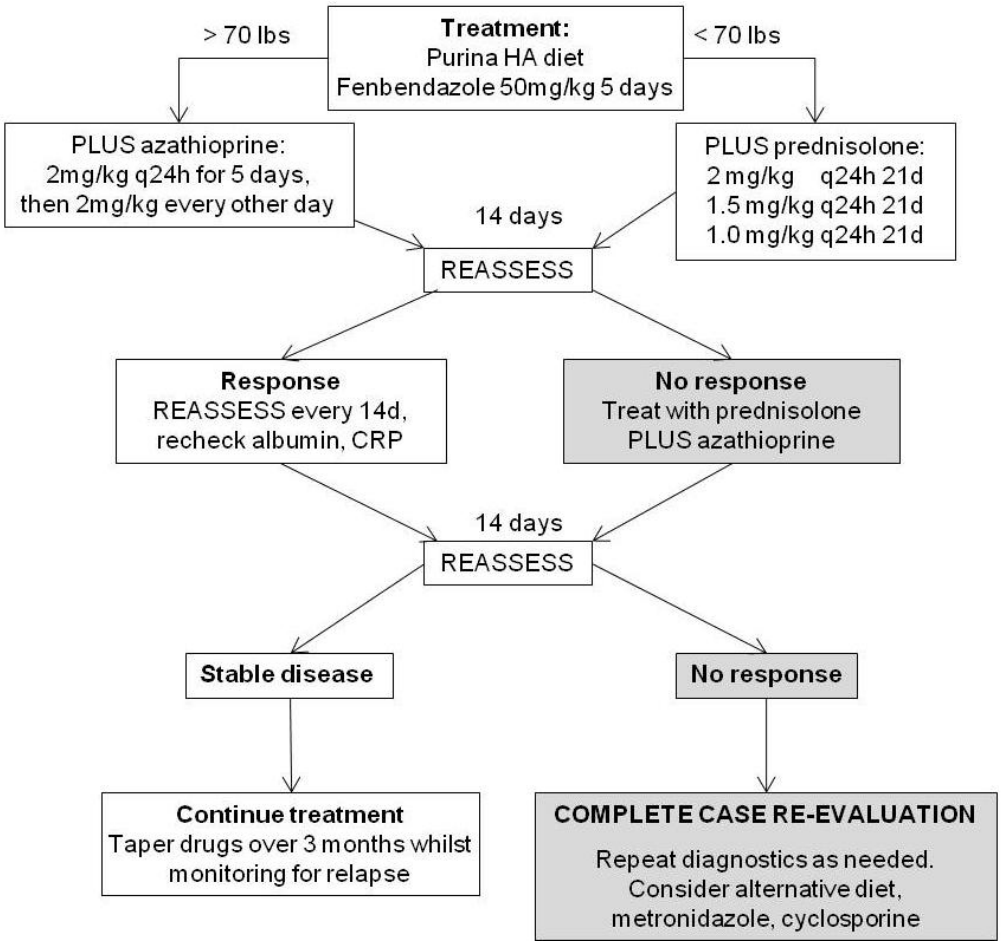


Figure 6.2: Treatment flowchart for dogs with albumin <2.0 g/dl

- *Outcome*

For maximal consistency, reassessment in every case was undertaken by the same investigator (Craven), by email or telephone communication every 14 days. Dogs that had shown improvement by a reduction in clinical signs by 50% or more were maintained on current treatment and reassessed every 14 days thereafter. Dogs that were deemed to have a poor response (estimated <25% response) or those that had responded but relapsed, or reached a plateau of response and not complete remission, entered the next phase of treatment, as per Figures 6.1 and 6.2. Dogs were ultimately classified as 'Food Responsive' (FR), 'Antibiotic Responsive' (AR), and 'Steroid Responsive' (SR).

- *Control dogs*

Small intestinal biopsies were collected from dogs anesthetized for non-GI disease to serve as controls. This group included Labrador Retrievers and hound dogs bred to study hip dysplasia and anesthetized for radiographs of their hips. In order to reduce bias due to environmental factors in the control population, intestinal biopsies from domestic pet dogs of various breeds were also included. These were obtained from healthy adult dogs whose owners (veterinary students and residents) elected to participate with their pet in an endoscopy training program (approved by Cornell University Institutional Animal Care and Use Committee (IACUC# 2009-0007 and 2007-101).

Sample collection and analysis

- *Serum*

At the time of clinical investigation, aliquots of serum from IBD-affected dogs were obtained for CRP measurement. In healthy dogs, pre-anesthetic CBC and chemistry profiles were performed, and unused serum was stored. Serum samples were stored at -80ºC and underwent batched analysis of CRP at the end of the study period.

- *Intestinal biopsy collection.*

Endoscopic biopsies were collected with informed consent from the duodenum as part of the routine diagnostic workup of dogs with chronic GI signs, into culture media (Luria Bertani broth), formalin, for quantitative histology and fluorescence in situ hybridization (8-12 biopsies), and a sterile Eppendorf tube containing tissue lysis buffer for 16S rDNA and pyrosequencing (2-3 biopsies).

- *Histology*

Formalin-fixed, paraffin embedded sections of intestinal mucosa were blindly evaluated by a board-certified veterinary pathologist and scored according to the guidelines of the WSAVA International GI Standardization Group[34]. Morphological features villus blunting, epithelial injury, crypt distention, lacteal dilation, and mucosal fibrosis were graded as normal (score), mild (1), moderate (2) and marked (3). Inflammation severity was graded by assessment of intraepithelial lymphocytes, lamina propria

lymphocytes, neutrophils, and eosinophils and graded identically from normal to marked. The total score was summed to allow group comparisons.

- *Fluorescence in situ hybridization* (FISH)

Formalin-fixed, paraffin-embedded archival intestinal biopsies were recut to 4 micron thickness and mounted on Probe-On Plus slides (Fisher Scientific, Pittsburgh, PA) for screening with a eubacterial 16S rDNA probe (EUB-338; GCTGCCTCCCGTAGGAGT).[20] Slides were deparaffinized by passage through xylene, 100% alcohol, 95% ethanol, and finally 70% ethanol (20 min) and air-dried. FISH probes 5' labeled with Cy3 or 6-FAM (Integrated DNA Technologies, Coralville, IA) were reconstituted with sterile water to a working concentration of 5 ng μl^{-1} with hybridization buffer (20 mM Tris-HCl, 0.1% sodium dodecyl sulfate [SDS], 0.9% NaCl [pH 7.2]). Hybridization with 30 µl of DNA probe mix in a hybridization chamber occurred at 46°C overnight. Slides were washed in hybridization buffer minus SDS at 48°C for 30 min, before final washing in phosphate-buffered saline (PBS), and mounted when dry with a ProLong antifade kit (Molecular Probes Inc., Eugene, OR). Sections were examined on a BX51 (Olympus America, Melville, NY) epifluorescence microscope, and images were captured with a Zeiss Axiocam or Olympus DP-7 camera, respectively. Slides spotted with *E. coli*, *Staphylococcus* spp. and *Streptococcus* spp. were used as positive controls. Probe specificity was additionally evaluated by counter-staining with the irrelevant probe non-EUB-338 (ACTCCTACGGGAGGCAGC).

- *Pyrosequencing*

Intestinal biopsies in tissue lysis buffer (Qiagen ATL buffer) were DNA extracted using a modified phenol chloroform technique. Pyrosequencing was based upon the bTEFAP method as described previously.[35-37] DNA was amplified using 27F-519R primers, labeled with linkers and tags, and pyrosequencing performed based upon manufacturer's instructions for Titanium chemistry FLX sequencing (Roche Applied Science, Indianapolis, IN). Data were curated so that only high quality sequence reads (Phred20) were used: sequences < 200bp were depleted, sequences with degenerate base calls were depleted, singletons were depleted, and the final data annotated with BLASTn.

- *Microbial culture*

Intestinal biopsies collected into sterile culture media (Luria Bertani broth) were archived at -80ºC. Individual biopsies were ground with a sterile pestle and plated onto blood agar for aerobic and anaerobic culture. Resultant colonies were be identified by morphology and biochemical analysis and archived at –80°C.

RESULTS

Study population

The IBD affected population consisted of 16 adult dogs (9M, 7F), of various breeds and ages (median 9.9 yrs, range 1-14 yrs, Table 6.1). Fourteen

healthy control dogs, with no history of GI signs or other known chronic disease were included (8F, 6M, median age 7.5 yrs, range 2-10 yrs), consisting of 9 Labrador retrievers (housed together in a research facility), and 5 privately owned dogs (3 mixed breed, 1 Greyhound, 1 GSD).

Clinical and clinicopathological parameters

The median duration of clinical signs prior to diagnosis was 6 months, and the most frequent clinical signs were diarrhea (n=12), vomiting (n=7), and weight loss (n=7) (Table 6.1). The characteristics of the diarrhea were consistent with a small bowel origin in 9 dogs, and diffuse large and small bowel in 7 dogs, including hematochezia in 5. Ascites was present in 3 of 4 dogs that were panhypoproteinemic (A3, A6, A7, A10), with median albumin 1.6 g/dL (range 1.4-1.9) and median globulin 1.5 g/dL (range 1.2-1.6); and this was accompanied by: hypocholesterolemia in 3 of 4 (median 72 mg/dL, range 71-77); hypocalcemia in 4 of 4 (median 7.8 mg/dL, range 7.7-8.3); reactive thrombocytosis in 3 of 4, (median platelet count 522, range 510-814 thou/uL) and 2 of 4 also had decreased levels of ATIII (62% and 40%; normal >65%). Urine specific gravity was low in 4 dogs of the 11 in which it was measured, ranging from 1.007-1.018 with no obvious cause. Clinicopathological parameters are summarized in Table 6.1. Folate and cobalamin were decreased in only 1 dog (A4), folate was increased in one dog (A15), and exocrine pancreatic insufficiency was excluded by TLI measurement in all cases with compatible clinical signs. A SNAP test for canine pancreatic lipase was performed in two dogs, and was negative.

Table 6.1: Signalments, historical, clinicopathological and outcome data for affected dogs. Abnormal values are shown in bold. ‘UNKN’ indicates parameter unknown.

Dog ID	SIGNALMENTS				CLINICAL SIGNS											CLIN-PATH PARAMETERS								CURRENT DIET (at presentation)	OUTCOME
	BREED	Sex	Age (yrs)	Bodyweight (kg)	Duration csx (mths)	Vomiting	Diarrhea	Weight Loss	Bleeding	Ascites	Anorexia	Lethargy	Tenesmus	Frequency	Mucus	ALB (3.1-4.1 g/dL)	GLOB (1.9-3.6 g/dL)	Chol (124-335 mg/dL)	Ca (9.3-11.6 mg/dL)	USG	HCT (42-57%)	WBCs (6.2-14.4 thou/uL)	Plts (179-483 thou/uL)		
A1	Boxer	ME	1.0	27.9	12		x				+/-	x	x	x		3.7	2.4	293.0	11.0	1.041	43.0	9.9	240.0	PURINA	**FA**
A2	Labrador	MN	10.5	27.2	6		x									3.3	2.3	207.0	9.9	1.040	43.0	**5.0**	217.0	HILLS	**FR**
A3	Labrador	MN	12.0	32.1	12		x	x			+/-					**1.9**	**1.3**	153.0	**8.3**	UNKN	41.0	11.0	**510.0**	EUKANUBA	**FR**
A4	St Bernard	FN	2.0	43.5	18	x	x				+/-					4.0	2.5	185.0	11.4	UNKN	52.0	7.9	321.0	HILLS	**FR**
A5	Irish Setter	ME	1.0	25.8	6		x	x	HC			x				3.2	2.1	127.0	10.3	1.052	41.0	21.4	**789.0**	NUTRO	**FR**
A6	X breed	MN	14.0	4.0	0			X		x	x					**1.4**	**1.6**	**71.0**	**7.9**	1.060	42.0	11.3	**552.0**	IAMS	**SR**
A7	Sheltie	MN	11.0	10.1	2		x	x		x	x	x				**1.5**	**1.2**	**72.0**	**7.7**	1.050	**30.0**	8.4	349.0	EUKANUBA	**SR**
A8	LabradorX	FN	9.7	27.5	6	x	x				+/-	x				3.5	2.4	258.0	10.3	**1.015**	48.0	7.7	259.0	PURINA	**FR**
A9	Malamute	FN	12.0	29.0	24	x	x	x								3.6	2.2	60.0	10.8	1.018	41.0	7.7	226.0	HILLS	**FR**
A10	Yorkshire Terrier	FN	4.0	3.0	1			x		x	x	x				**1.6**	**1.6**	**77.0**	**7.7**	1.036	45.0	18.5	**814.0**	HILLS	**SR**
A11	WHWT	MN	2.0	9.0	6	x	x		HC							3.6	2.7	264.0	11.0	**1.010**	52.0	7.4	340.0	HILLS	**FR**
A12	English Bulldog	FN	9.8	30.9	60			x	HC							3.3	4.0	**342.0**	10.6	**1.007**	59.0	9.1	410.0	HILLS	**FA**
A13	X breed	MN	2.5	9.4	3	x	x		HC						x	3.9	2.4	246.0	10.8	1.060	54.0	12.0	256.0	PURINA	**FR**
A14	X breed	FN	10.0	20.4	4						x	x	x			4.0	2.8	198.0	11.1	UNKN	54.0	6.7	299.0	HILLS	**FA**
A15	Bichon Frise	FN	13.0	7.9	12	x	x		HC							3.9	3.2	187.0	10.2	UNKN	48.0	13.1	369.0	HILLS	**FR**
A16	Pomeranian	MN	13.0	4.4	36	x	x									3.3	3.0	185.0	10.3	UNKN	44.0	13.6	453.0	NUTRO	**FR**

Hematological abnormalities (Table 6.1), included leukocytosis due to mild mature neutrophilia (dogs A5 and A10), mild eosinophilia (A5 and A16). In dog A7, a moderate but stable normochromic, normocytic, non-regenerative anemia was present, hematocrit 30% (ref. range 42-57%). Anemia of this magnitude was not explained purely by chronic disease, and given that A7 was also hypoalbuminemic, GI hemorrhage may have contributed although was not supported by erythrocyte morphology. Hypoadrenocorticism was considered as a differential diagnosis in 9 cases, and was excluded by measurement of baseline cortisol in 3 dogs and by performing an ACTH response test in 6. C-reactive protein was mildly elevated in dog A8 (15.8 ug/ml), but was <5 ug/ml in all other cases (reference range 0-10 ug/ml).

In control dogs, the results of CBC, renal parameters, hepatic enzymes (ALT and ALP), electrolytes (Na, K, Cl), calcium, blood glucose, and albumin and globulin were within reference limits.

Fecal analysis

On fecal flotation, *Isospora* cysts were discovered in one case, and were not considered to be significant. Fecal culture yielded *Salmonella* in dog A1, which had experienced waxing and waning diarrhea for approximately one year. Antimicrobial therapy was not deemed necessary since the dog was clinically well, and repeat fecal cultures twice (2 weeks and 4 weeks later on 3 samples from 3 consecutive days) were negative for *Salmonella*. Since the dog's clinical signs remained unchanged, this was thought to be an incidental finding. *Giardia* and *Cryptosporidium* ELISA, performed in 10 cases, were negative.

Imaging

Thoracic radiographs, performed in 8/16 dogs, revealed no significant findings. Abdominal ultrasonography was performed in all dogs and showed nonspecific changes in 12/16 cases, such as occasional splenic or hepatic nodules that were considered likely to be benign. In the 4 hypoalbuminemic dogs, all had evidence of hyperechoic speckling of the small intestinal wall and ascites was visualized and sampled using ultrasound guidance in 3 of 4, confirming presence of a transudate.

Endoscopy

Upper GI endoscopy and duodenal biopsy was performed in all cases. Gross changes were usually mild and nonspecific, including mucosal hyperemia, friability, and prominent lymphatics in some cases. Mucosal ulceration, foreign bodies and masses were not reported. Colonoscopy and biopsy was also performed in animals with lower GI signs (n=7).

Histology

In affected dogs, histologic severity was graded as mild in 4/16 dogs, and moderate in 12/16 (maximum possible score 27). Summed histology scores ranged from 2-11, median 6.5 (Table 6.2a, Figure 6.3). In healthy dogs (n=16), pathology was graded as normal in 2, mild in 10, and moderate in 2 (range 1-12, median 3.0, Table 6.2b). Summed scores were significantly higher in the affected versus control group ($p<0.02$). Varying degrees of colitis were also present in the dogs that underwent colon biopsy. Histology scores were not significantly associated with serum C-reactive protein levels.

Table 6.2 a: Intestinal histology scores, outcome and potential disease severity markers in affected dogs, designated A1-16. Abnormal values shown in bold.

DOG	Total histology score	Severity	Response	Albumin (3.1-4.1 g/dL)	CRP (0-10ug/ml)	Fibrinogen (147-479 mg/dL)	Platelets (179-483 thou/uL)	ATIII (65-145%)
A1	3	mild	FA	3.7	0.10	-	240.00	-
A2	7	moderate	FR	3.3	2.17	-	217.00	-
A3	11	moderate	FR	**1.9**	0.04	**662**	**510.00**	**62**
A4	9	moderate	FR	4.0	1.75	-	321.00	-
A5	2	moderate	FR	3.2	0.12	-	**789.00**	-
A6	5	mild	SR	**1.4**	2.70	397	**552.00**	**40**
A7	8	moderate	SR	**1.5**	0.02	**583**	349.00	76
A8	3	mild	FR	3.5	**15.77**	-	259.00	-
A9	5	moderate	FR	3.6	0.13	-	226.00	-
A10	10	moderate	SR	**1.6**	2.79		**814.00**	-
A11	9	moderate	FR	3.6	0.48	-	340.00	-
A12	10	moderate	FA	3.3	2.16	-	410.00	-
A13	6	moderate	FR	3.9	0.01	-	256.00	-
A14	6	moderate	FA	4.0	3.81	-	299.00	-
A15	7	moderate	FR	3.9	1.60	-	369.00	-
A16	4	mild	FR	3.3	0.16	**882**	453.00	101

Table 6.2 b: Intestinal histology scores in healthy dogs, designated H1-14.

DOG	Total histology score	Histologic severity
H1	5	mild
H2	5	mild
H3	5	mild
H4	1	normal
H5	10	moderate
H6	2	mild
H7	4	mild
H8	3	mild
H9	12	moderate
H10	1	normal
H11	1	mild
H12	3	mild
H13	2	mild
H14	3	mild

Clinical phenotype

Ten dogs (62%) were food responsive (FR), 3 (19%) were food and antibiotic responsive (FAR) and 3 (19%) were steroid responsive (SR). Food responsive dogs had significantly higher histology scores relative to controls ($p<0.05$) and there were insufficient numbers in the other treatment groups to enable meaningful statistical analysis (Figure 6.3). The two dogs with peripheral eosinophilia were both FR, but we did not observe overrepresentation of eosinophils on histology in FR dogs. In general, FR dogs demonstrated a positive response within seven days of implementing dietary change.

One FR dog (A3) was hypoalbuminemic dogs and treatment of this dog did not follow the protocol for hypoalbuminemic animals. This dog was non-ascitic and relatively stable, thus dietary change alone (to Purina HA®), was implemented whilst awaiting histology results. The dog exhibited excellent improvement on diet alone and treatment with prednisolone was unnecessary. Within 7 days his albumin level had increased from 1.9 to 2.6 g/dL, followed by normalization within 4 weeks. In the remaining 3 hypoalbuminemic dogs (A6, A7, A10), treatment with prednisolone was begun at the same time that dietary change was implemented because of the severity of clinical signs and ascites present. Thus, whether or not these dogs would also have responded to dietary change alone is unknown. They did, however, respond completely to steroid administration ('SR,' steroid responsive). Three dogs were partially FR, but were experiencing an unacceptable level of clinical signs and responded completely when treatment with tylosin was introduced ('FAR', food and antibiotic responsive).

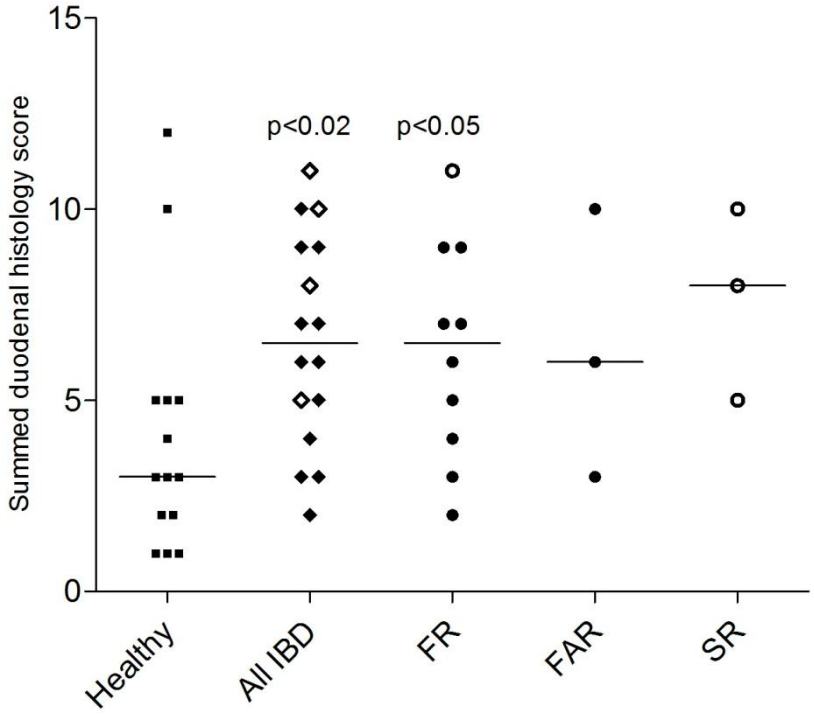


Figure 6.3: Scatter plot of summed WSAVA histology scores for IBD-affected and control dogs. Bars represent the median score, and hollow data points indicate hypoalbuminemic animals (FR- food responsive; FAR - food and antibiotic responsive; SR- steroid responsive). Affected dogs had significantly higher scores than controls ($p<0.02$, Mann Whitney) and when analyzed by response to treatment, scores for FR dogs were higher than controls ($p<0.05$, Mann Whitney).

Pyrosequencing

Bacterial 16S rDNA pyrosequencing of duodenal biopsies yielded a total of 520 different bacterial species (440H, 392A) and analysis of species diversity by rarefaction revealed that IBD affected dogs had lower overall species diversity than controls ($p<0.05$).

Overview at Class level of median relative sequence proportions from healthy dogs revealed overrepresentation of Class *Bacteroidetes* compared to all other Classes in this group ($p<0.0001$, 2 way ANOVA, Figure 6.4). In IBD dogs, Class *Bacteroidetes* was enriched compared to *Actinobacteria* ($p<0.01$), *Mollicutes* ($p<0.001$), *Betaproteobacteria*, *Alphaproteobacteria* ($p<0.0001$), *Fusobacteria* ($p<0.01$), *Spirochaetes* ($p<0.0001$), and *Sphingobacteria* ($p<0.001$). Proportions of *Gammaproteobacteria* were also significantly greater than *Spirochaetes* ($p<0.05$) in IBD dogs. Comparisons between healthy and IBD at Class level (t test) revealed lower proportions of *Bacteroidetes* ($p<0.05$) in IBD, with enrichment of *Bacilli* ($p<0.05$) and *Betaproteobacteria* ($p<0.05$).

Evaluation of total sequence number by response to treatment (Figure 6.5 a) revealed significant differences between groups H, FR, SR, and FAR (Kruskal Wallis, $p<0.003$) and lower sequence numbers in FR compared to H ($p=0.007$).

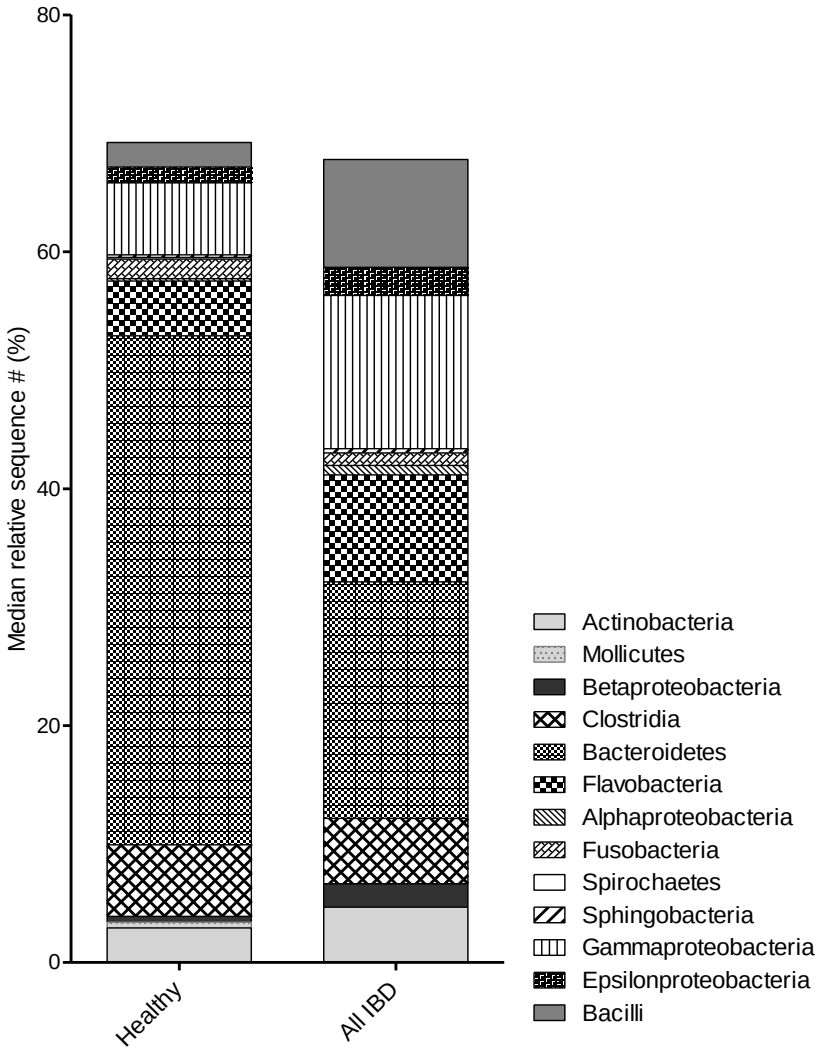
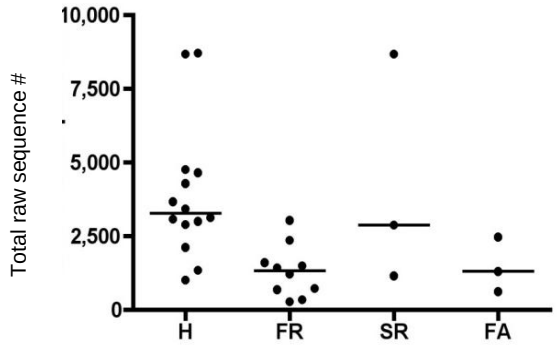


Figure 6.4: Class-level composition and comparison of duodenal microflora as determined by 16S rDNA eubacterial pyrosequencing of healthy dogs (n=14) and all IBD-affected dogs (n=16).

(a).



(b).

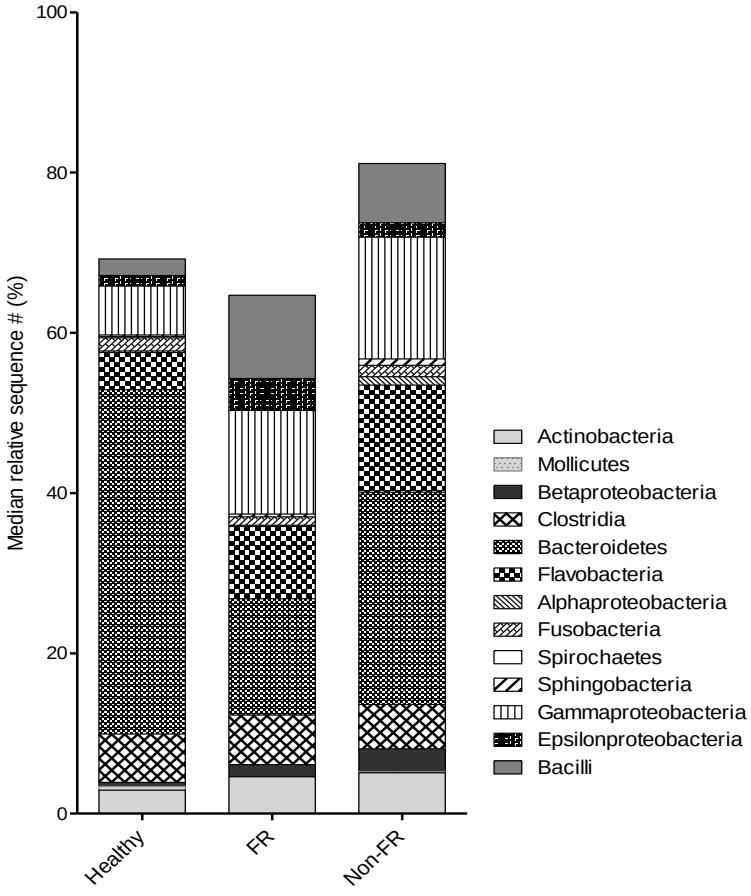


Figure 6.5: **(a).** Analysis of duodenal 16S rDNA pyrosequencing total sequence # by response to treatment reveals significant differences between groups (Kruskal Wallis, $p<0.003$) and lower numbers in FR than H ($p=0.007$) **(b).** Class-level composition and comparison of flora as determined for H, FR, and non-FR.

Given that the majority of animals were FR, further analysis of bacterial Class in the IBD dogs according to treatment was performed by grouping non-food responders together (i.e. FR vs non-FR, Figure 6.5 b). Significant differences in Class proportions in non-FR included enrichment of *Bacteroidetes* in comparison with Classes *Mollicutes* ($p<0.01$), *Betaproteobacteria* ($p<0.05$), *Alphaproteobacteria* ($p<0.01$), *Spirochaetes* ($p<0.01$), *Epsilonbacteria* ($p<0.05$), and *Sphingobacteria* ($p<0.01$). No significant differences in class proportions were observed within group FR (2 way ANOVA). When we compared between FR and non-FR at Class-level (t test), there were also no significant differences.

When the dataset is scrutinized at a higher taxonomy level (Order) we observed greater relative proportions in IBD versus H for: Gram positive (G+) *Actinomycetales*,*** Gram negative (G-) *Burkholderiales*,** G+ *Lactobacillales*,** G- *Erysipelotrichales,** G- Rhizobiales*, G- *Xanthomonadales*, and depletion of G- *Bacteroidales** (***$p<0.001$, **$p<0.01$, *$p<0.05$, Mann Whitney). On balance, this indicates a more diverse G- population in dogs with IBD. (Table 6.3, Figure 6.6).

When we compare FR versus H at Order-level, FR dogs harbor greater relative proportions of *Actinomycetales,*** *Burkholderiales*,** *Lactobacillales,** *Erysipelotrichales*,* *Rhizobiales,** and *Xanthomonadales,** and depletion of *Bacteroidales**** (***$p<0.001$, **$p<0.01$, *$p<0.05$, Mann Whitney, Figure 6.6).

Table 6.3: 16SrDNA eubacterial pyrosequencing data analysed by Order, for 14 healthy controls and 16 IBD-affected dogs.

ORDER	HEALTHY														TOTAL	AFFECTED															TOTAL
	H1	H2	H3	H4	H5	H6	H7	H8	H19	H10	H11	HI2	H13	H14		A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	A14	A15	A16	
Actinomycetales	59	64	51	40	2	14	5	86	25	38	15	30	1	45	**475**	51	3	8	40	30	28	111	18	22	318	50	140	58	60	9	80	**1026**
Bacillales	7	8	10	2	32	3	0	11	5	22	2	1	1	12	**116**	26	0	3	9	0	5	1	73	2	13	3	12	13	5	1	2	**168**
Bacteroidales	3052	322	2670	1376	30	32	834	565	734	266	69	592	527	796	**11865**	50	4	335	29	48	759	99	210	3	1169	131	428	76	46	38	204	**3629**
Burkholderiales	54	39	33	8	9	9	1	22	17	7	89	6	2	24	**320**	57	2	2	9	44	9	72	16	16	30	24	14	22	10	4	18	**349**
Campylobacterales	9	1123	33	20	107	1824	0	16	16	7	1	9	5	32	**3202**	1	3	79	4	2	17	9	1	498	49	16	8	33	1	2	34	**757**
Clostridiales	826	87	581	141	25	14	58	425	139	56	44	69	44	214	**2723**	25	10	76	23	3	99	93	39	13	154	56	73	219	32	65	139	**1119**
Coriobacteriales	476	6	12	121	0	0	17	9	14	4	24	21	8	89	**801**	1	2	0	8	0	109	7	7	1	1	1	4	69	0	0	2	**212**
Desulfuromonadales	5	0	5	2	0	1	0	9	7	6	4	11	1	3	**54**	1	1	9	1	0	0	0	7	0	59	0	0	0	8	1	1	**88**
Enterobacteriales	24	166	49	8	62	10	3	244	8	379	5	4	4	65	**1031**	15	12	2	10	9	24	70	23	97	231	13	18	17	4	8	22	**575**
Erysipelotrichales	95	1	85	3	1	4	15	16	8	8	12	27	22	14	**311**	1	0	0	0	0	12	0	1	2	0	2	1	27	0	0	2	**48**
Flavobacteriales	537	318	308	279	1037	55	58	142	173	388	58	811	77	311	**4552**	233	0	141	77	113	104	3	139	7	3105	190	61	254	111	32	59	**4629**
Fusobacteriales	1070	21	296	27	2	5	3	121	27	15	1	17	3	91	**1699**	1	2	1	8	0	165	0	11	0	46	8	115	2	0	2	31	**392**
Lactobacillales	68	13	800	12	3	19	3	55	7	13	6	14	1	11	**1025**	13	2	9	40	43	20	36	37	18	5	39	20	36	16	5	69	**408**
Mycoplasmatales	39	19	223	83	58	11	1	2	26	61	1	18	0	3	**545**	0	0	134	1	0	58	0	1	15	59	29	30	3	0	0	0	**330**
Neisseriales	78	1	61	2	3	28	1	23	4	7	0	4	5	1	**218**	7	0	2	10	0	0	3	13	3	5	107	112	8	3	1	3	**277**
Pasteurellales	195	66	474	15	14	21	6	58	75	22	0	34	6	45	**1031**	23	0	28	14	15	18	53	40	9	18	606	68	107	3	2	28	**1032**
Pseudomonadales	98	50	62	73	1	1	65	57	181	211	23	72	28	12	**934**	56	0	10	20	18	3	32	13	3	8	94	76	49	16	0	0	**398**
Rhizobiales	38	12	63	3	5	2	5	8	0	4	28	5	0	2	**175**	17	4	0	18	2	8	21	18	11	8	54	5	4	4	0	9	**183**
Rhodobacterales	0	0	1	2	0	0	0	0	1	0	124	0	0	1	**129**	14	0	0	0	0	0	0	0	2	1	0	0	0	5	0	1	**23**
Sphingobacteriales	187	11	45	28	6	5	4	36	20	12	257	178	6	0	**795**	11	0	35	16	0	53	22	10	16	31	3	17	323	4	4	9	**554**
Vibrionales	55	0	72	0	30	0	0	0	0	0	0	0	0	0	**157**	0	0	0	0	0	0	24	0	1	0	0	0	0	6	0	0	**31**
Xanthomonadales	13	5	3	5	83	5	0	9	9	12	2	2	0	0	**148**	204	0	2	18	7	1	30	21	0	53	18	4	5	7	6	11	**387**
TOTAL	**6985**	**2332**	**5937**	**2250**	**1510**	**2063**	**1079**	**1914**	**1496**	**1538**	**765**	**1925**	**741**	**1771**	**32306**	**807**	**45**	**876**	**355**	**334**	**1492**	**686**	**698**	**739**	**5363**	**1444**	**1206**	**1325**	**341**	**180**	**724**	**16615**

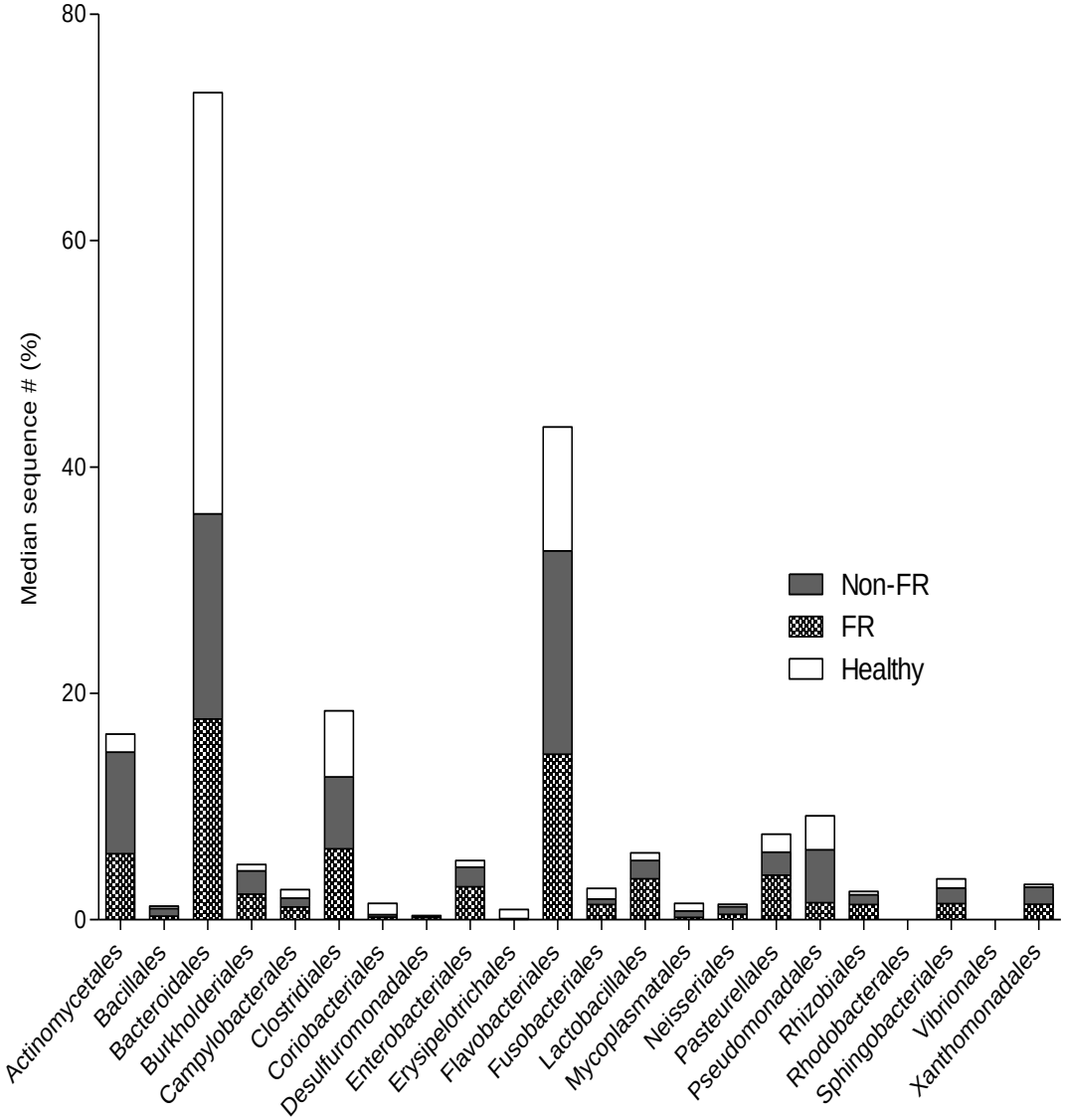


Figure 6.6: Order-level median relative sequence proportion (%) from 16S rDNA eubacterial pyrosequencing of duodenal biopsies in 14 healthy controls and 16 IBD-affected dogs (FR- food responsive, non-FR non-food responsive).

FISH analysis

Eubacterial FISH analysis revealed very few mucosa-associated bacteria in all dogs, and bacterial invasion was not detected (Figure 6.7). Too few bacteria were present for meaningful quantification, and subjectively we observed no differences according to histologic severity or outcome.

Microbial culture

Culture of duodenal biopsies yielded no growth for all control and affected dogs.

DISCUSSION

This study has performed in-depth evaluation of the canine duodenal microbiome and analyzed microbial imbalances according to clinical phenotype in order inform management of IBD. We have shown that duodenal inflammation results in decreased diversity of the mucosal microflora, and altered relative proportions of the dominant floral groups. Specifically, duodenal inflammation is associated with enrichment of *Actinomycetales*, *Burkholderiales*, *Erysipelotrichales*, *Rhizobiales*, *Xanthomonadales* and *Lactobacillales.* Collectively, these changes indicate a broad-based expansion of Gram negative species in canine IBD. However we also discovered marked selective depletion in Gram negative *Bacteroidales* in IBD-affected dogs, which is in line with previous work.[38] This was particularly evident in the FR

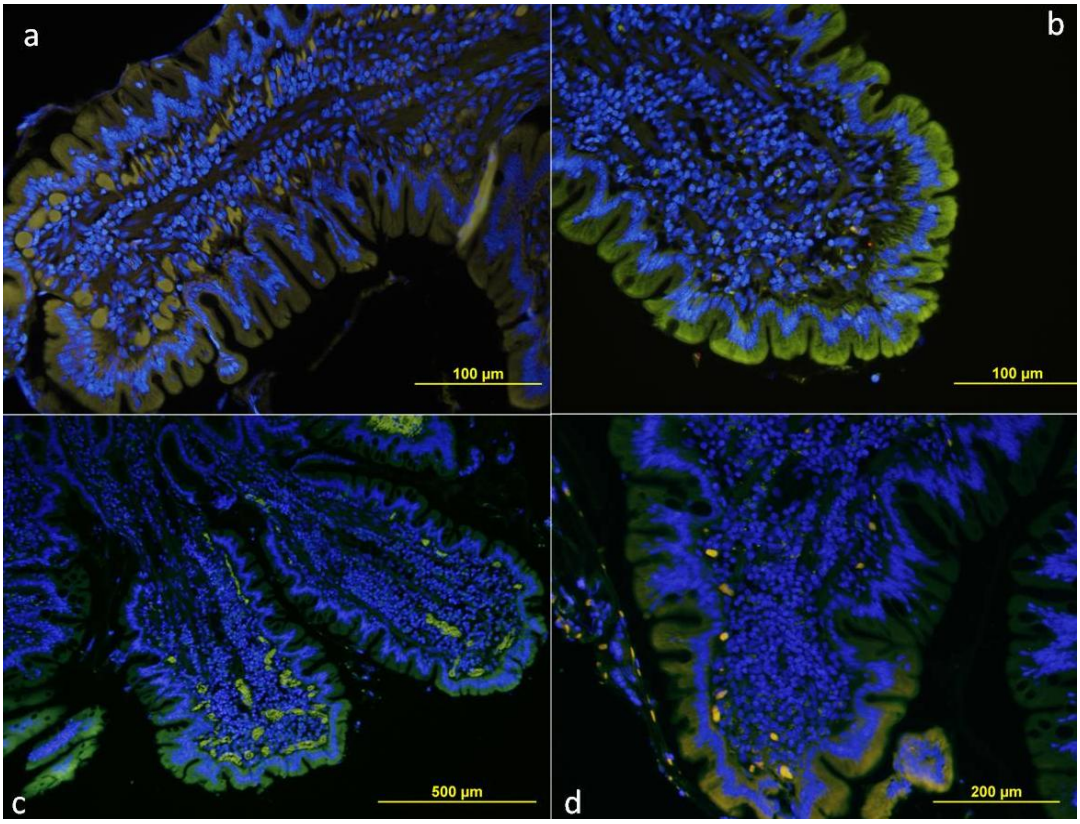


Figure 6.7: Eubacterial FISH (EUB338-Cy3 (red), Non-EUB3386FAM (green) with DAPI nuclear counterstaining (blue) in healthy dogs H1, H2 **(a,b)** and IBD-affected dogs A4, A14 **(c,d).** Very scant mucosa-associated bacteria were present in both groups, and bacterial invasion was absent. Note the increased cellular infiltrates in (d), and stunted villi in (c)

group, and warrants further evaluation of *Bacteroidales* depletion as a potential marker of IBD and clinical phenotype in larger treatment groups.

Our conclusion that the canine intestinal microbiome differs in health and disease mirrors the situation in people, where the dominant flora in IBD patients differs from healthy patients.[20,43-45] In contrast to our findings, Gram- enrichment in CD is dominated by *Enterobacteriaceae*, alongside depletion in G+ *Firmicutes*, especially *Faecalibacterium praustnitzii,* which serves epithelial health by butyrate production.[20,39,46] Earlier studies in IBD-affected dogs using bacterial 16S clone libraries have shown similar findings to this, including decreased species richness, and enrichment with Gram negative spp. such as *Enterobacteriaceae* and depletion of *Bacteroidetes* in IBD.[33,38] We do not address the causal relationships between these microbial imbalances and mucosal inflammation, and the unresolved issue is whether the microbial alterations in dysbiosis are the primary driving force underlying inflammation, or a secondary change. Potential mechanisms of deleterious effects due to dysbiosis are poorly characterized, but seem likely when we consider that the resident microbiome performs a multitude of tasks in maintaining mucosal immune homeostasis and metabolic functions. Perturbations in the dominant bacterial groups may therefore be expected to adversely affect intestinal health.[12,47] Increasing evidence supports a mechanistic role for dysbiosis in CD pathogenesis, which similarly involves an altered balance of aggressive Gram- versus protective Gram+ bacteria.[43,48-50] Clinical evidence in CD and UC patients suggests a pathogenic role for the microflora,[39 40] specifically implied by clinical responses to antibiotics, pre- and probiotics.[28-30,40,41]

Despite these marked perturbations in the qualitative composition of the microflora, we found surprisingly few bacteria on FISH analysis of healthy and affected dogs. The paucity of mucosa-associated organisms observed on FISH may explain the negative microbial cultures of duodenum. This apparent discordance between culture-independent and culture dependent methodology likely reflects loss of the mucus layer in biopsy processing, as well as the relatively low level of colonizing and cultivable bacteria in the duodenum. This highlights the benefits of developments in metagenomic technologies for advancing our knowledge in this area.

Grading of IBD histology is an issue of contention in veterinary medicine. While histologic severity scores were significantly greater in IBD versus H, it was notable that 2 controls, H5 and H9 (research Labradors, housed in a controlled environment) had scores (10 and 12 respectively) comparable to affected dogs. Neither dog had clinical signs of GI disease, and were housed in a controlled research environment. They had experienced no recent dietary change, had zero opportunity for scavenging or foreign body ingestion, and no other obvious cause of enteritis. Careful scrutiny of the biopsies indicated that these high scores were largely attributable to high levels of cellular infiltration. The relationship of cellular infiltration to inflammation is contentious, with some studies showing similar numbers of infiltrates before and after successful treatment of IBD in dogs.[5] It is possible that these dogs could have had developing, or subclinical IBD, or very focal pathology. Upon further monitoring, they did not progress to develop clinical signs of GI disease. It is debatable whether or not these dogs should have been included in the control group. However, 12 of the 14 healthy dogs

exhibited subclinical duodenal pathology, as has been documented by other authors.[51] Since diagnosis of IBD relies upon the presence of consistent clinical signs as well as histologic abnormalities, these dogs were considered to represent a realistic spectrum of 'controls.' Exclusion of these two dogs from statistical analyses of 16S rDNA pyrosequencing did not change the significant microbial imbalances.

An unexpected finding was the complete response of dog A3 to dietary change alone. This dog had moderate small bowel diarrhea, weight loss, panhypoproteinemia, moderate lymphangiectasia and third highest histology score (11). This argues for evaluation of dietary trial prior to aggressive immunosuppression even in severely affected and hypoalbuminemic cases. We anticipated that serum CRP would correlate with histologic severity, but actually only one affected dog had slightly raised CRP. Our results are consistent with other studies that show no association of CRP with histologic severity.[5,52]

A major finding of the current study was that most of the dogs were responsive to a commercial hydrolyzed soy protein diet (62.5%). The rationale for using a hydrolyzed diet is that the hydrolytic process produces the protein into smaller, less antigenic polypeptides that are less immunostimulatory.[54] This is a higher response rate than previously reported using a salmon/rice elimination diet (Purina LA, 55%, 39/78 FR[5]). In that study, a higher proportion of dogs were steroid responsive, 27%, compared to 19% here. A more recent study evaluating a different commercial hydrolyzed soy protein diet found a yet higher response rate response rate of 89% (n=16/18, Royal Canin Hypoallergenic® diet).[53] The specific dietary ingredients in commercial foods

that may be associated with development of mucosal inflammation are unknown. Dietary rechallenge was not rigorously performed here, mainly due to owner reluctance given the excellent outcome in responding dogs. However, 4 cases are known to have relapsed when the diet was changed for cost reasons, and responded equally well when it was reintroduced in all 4.

In people, adverse reactions to food are known to occur in response to to constituents such as e.g. casein, lactose, fructose, sucrose, citric acid and gluten.[55-57] Gluten intolerance is best known as celiac disease, but reactivity to gluten is increasingly recognized in patients with other enteropathies, including IBD and irritable bowel syndrome (IBS).[58,59] Future directions that may help to elucidate the offending antigens include re-introduction of single constituents of commercial diets after establishing food responsiveness, e.g. gluten, carageenan, poultry proteins, fructose. Immunohistochemical study of intestinal biopsies for antibodies directed at gliadin and transglutaminase may also be worthwhile.

In summary, we discovered that responsiveness to food in dogs with IBD is demarcated by marked depletion in *Bacteroidetes* sequences ($p<0.001$). In people, enrichment and mucosal adherence of *Bacteroidetes* has also been associated with IBD,[60] but this is an inconsistent finding and appears to vary according to the anatomical site of disease (ileum versus colon). [12] The significance of, and reasons for this relative depletion in dogs are unclear, but may involve perturbations in the mucosal microenvironment that inhibit anaerobic proliferation, luminal pH, or affect substrate availability. It is not clear how food responsiveness, mucosal inflammation and dysbiosis interrelate. However, if we assume a pathogenic role for dysbiosis, intestinal

bacteria depend on dietary and host drive nutrients for survival, andso it is not surprising that bacterial composition is profoundly influenced by diet. We postulate that in addition to removing potentially immunogenic antigens, dietary change may dampen dysbiosis by impacting bacterial substrates, growth conditions, and reducing inflammation. Our findings indicate that *Bacteroidetes* depletion appears to holds promise as a marker of canine IBD. Further evaluation in a larger group of dogs with greater diversity in outcome is required to ascertain whether this predicts treatment responses (see Appendix 2 for updated status at the time of writing).

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CHAPTER 7

Wolinella Rather Than *Helicobacter* Spp. is the Predominant *Helicobacteraceae* in the Canine Oral Cavity.

ABSTRACT

The presence of *Helicobacter* spp. DNA in saliva and dental plaque of >70% of dogs suggests that contact with dogs is a risk factor for infection of non-pylori Helicobacter spp. to people, and supports an oral route of transmission in dogs. Our objective was determine the *Helicobacter* spp. present in the canine oral cavity and their relationship to gastric *Helicobacter* spp., in order to better define the potential for dog-human and dog-dog transmission.

PCR-based screening for *Helicobacter* spp. was undertaken in the oral cavities of 28 dogs. Comparative analysis of *Helicobacteraceae* 16S rDNA clone libraries from the oral cavity and stomach of a subset of 8 dogs (5 vomiting, 3 healthy) positive for *Helicobacter* spp. was performed by PCR screening. *Helicobacteraceae* DNA was identified in the oral cavity of 24/28 dogs. Analysis of cloned 16S rDNA amplicons from 8 dogs revealed that *Wolinella succinogenes* was the most prevalent (8/8 dogs) and abundant (91% of 57 clones) member of the *Helicobacteraceae* in the oral cavity. Only 2/8 dogs harbored oral *Helicobacter* spp., and one of those was co-infected with "*H. heilmannii*" and *H. felis* in stomach and saliva. Evaluation of oral cavity DNA with *Wolinella*-specific PCR was positive in 16/20 other dogs, totalling 24/28 *Wolinella* positive samples.

We conclude that *W. succinogenes*, rather than *Helicobacter* spp. is the dominant *Helicobacteraceae* in the canine oral cavity. The oral cavity of dogs does not appear to be a significant reservoir of non-pylori *Helicobacter* spp. of zoonotic importance.

INTRODUCTION

Spiral bacteria of the family *Helicobacteraceae,* principally *Helicobacter pylori,* colonize the stomach of approximately 50% of the world's human population[1] and are causally associated with chronic superficial gastritis, peptic ulceration, gastric adenocarcinoma and MALT lymphoma.[2-4] Gastric colonization by *Helicobacter* is also common in dogs (61-100%[5,6]), but the infecting species are a diverse group of non-*H. pylori Helicobacter* spp., including *H. bizzozeronii*, "*H. heilmannii*", *H. felis*, *H. salomonis, H. bilis*, *H. cyanogastricus* and *Flexispira rappini.*[5, 7-10] The consequences of *Helicobacter* infection in dogs are less defined, with infection linked to lymphoid hyperplasia and gastritis, rather than cancer and peptic ulceration.[8] Infection of people with non-*pylori Helicobacter* species, typically categorized as "*H. heilmannii,*" is reported in 0.25% to 6.0% of individuals and is also associated with gastritis and MALT lymphoma.[3,11,12] However, recent reports highlight the presence of *Helicobacter* spp., typically associated with the stomachs of dogs e.g. *H. bizzozeronii/salomonis*, rather than "*H. Heilmannii*" in up to 48 % of people with non-*H. pylori* infection.[10]

The high prevalence of non-*H.pylori Helicobacter* infection in dogs[5,6] raises the possibility that dogs are a potential reservoir for infection of people. The potential for zoonotic spread is supported by reports of increased risk of "*H. heilmannii*" infection in people in contact with dogs, cats, and pigs.[13] A questionnaire-based study of *Helicobacter*-positive human patients also revealed that 70.3% owned pets, compared to an overall incidence of 37%.[4] More direct evidence is provided by reports of similar strains of *Helicobacter* spp. being isolated from people and their pets.[14,15] However, the potential zoonotic risk of dogs for "*H. heilmannii"* infection has been challenged by the relatively low prevalence of "*H. heilmannii"* in dogs, and gastric colonization of dogs with "*H. heilmannii*" Types 2 and 4,[16] rather than Type I, which is the dominant subtype in people.[17]

The precise mode of transmission of *Helicobacter* spp. is unresolved in all species, with fecal-oral, oral-oral, and gastric-oral routes previously hypothesized.[2,7,18] The recognition of the oral cavity as an entry point for gastric *Helicobacter* spp. in humans was first demonstrated by Marshall et al (1985),[1] who ingested *H. pylori* and subsequently developed gastritis. *H. pylori* DNA has since been detected in the saliva, supragingival and subgingival plaque, oral mucosa and dorsal tongue of human patients, though detection rates vary from 0-90%.[19] Dogs are known to acquire *Helicobacter* infection at an early age through close contact with their mothers,[20] and recent studies have documented the presence of *Helicobacter* DNA in the saliva and dental plaque in 50% and 44.7% (n=38) of dogs, with over 70% of dogs positive in plaque or saliva.[21] As contact with oral secretions is the most plausible route

of zoonotic infection from dogs we sought to more critically examine the potential zoonotic risk posed by dogs by defining the *Helicobacter* spp. present in the canine oral cavity and their relationship to gastric *Helicobacter* spp.

METHODS

Animals and sampling:

Saliva and dental plaque from 28 pet dogs of varied signalments were collected into sterile phosphate buffered saline on ice. A subset of 8 dogs, (4M, 4F), ranging from 6 months to 14 years old (mean 6.8 yrs), underwent gastric biopsy (5 vomiting, 3 healthy) and biopsy samples were collected into sterile Eppendorf tubes on ice. Endoscopy and biopsy sampling were performed as part of clinical investigation with the owners' agreement. The study was approved by the Ethical Committee of the Veterinary School of the University of Milan.[21] DNA was extracted from oral and gastric samples as previously described [21,22] and stored at -80C.

PCR for *Helicobacter* spp.

Aliquots of DNA were PCR amplified with *Helicobacter* genus-specific primers, C97 and C05 (GCTATGACGGGTATCC and ACTTCACCCCAGTCGCTG respectively), generating 1200bp 16S rDNA gene amplicons[5, 23.] Briefly, samples were thawed on ice and 2µl was added to a 50µl volume containing 25pmol of each primer and 25µl of Taq polymerase Master Mix (QIAGEN Inc.). Samples were heated to 94°C for 10 min once, followed by 35 cycles of denaturation for 1 min at 94°C, primer annealing for

1.5 mins at 58°C, and extension at 72°C for 2 mins, with a final extension at 72°C for 15 mins in an Eppendorf thermocycler (Mastercycler gradient, Eppendorf GA, Hamburg, Germany). Negative controls in which the DNA extract was omitted were included with each reaction. The PCR products were visualized by agarose gel (1%) electrophoresis with ethidium bromide, in Tris-acetate EDTA buffer. The size of the expected fragment was compared to a 100bp reference marker (Fermentas Inc, Maryland, US).

Construction and analysis of 16S rDNA clone libraries

DNA from the oral cavity and stomach of 8 dogs positive for *Helicobacter* spp. on PCR (5 vomiting, 3 healthy) was amplified with primers C97/C05 PCR as described above. 1200-bp 16S rDNA products were purified using a Qiaquick PCR purification kit (QIAGEN Inc), cloned into the TA cloning vector, pGEM®-T Easy (Promega, Madison, Wisconsin, US), and introduced into *E. coli* DH5α. Plasmid DNA from up to 10 clones from each sample was purified using the Qiaprep Spin Miniprep Kit (QIAGEN Inc) and clones were sequenced at the Cornell University BioResource Center with M13 primers and AmpliTaq® DNA Polymerase (Applied Biosystems, Foster City, California, US), using an ABI 3700 automated DNA sequencer and ABI PRISM® BigDye™ Terminator Sequencing Kits. DNA sequences obtained with forward and reverse primers were extracted from flanking vector sequences using Sequencher software (Gene Codes Corp., Ann Abour, Michigan, US), and contiguous sequences were aligned in Sequencher. The identity of partial 16S rDNA sequences was determined by comparison to the sequence databases at the Ribosomal Database Project (RDPII: http://rdp.cme.msu.edu/) and the

NCBI database (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic analyses were conducted using the neighbor-joining method in MEGA version 3.1.[24]

PCR for *Wolinella*

On the basis of the results of 16S rDNA sequence analysis, we designed primers against *Wolinella*: WOL1, forward: AAAGAGCACGTAGGCGGC (position 316,294-316,272) and WOL2, reverse: CAGGATTCTATCAATGTCAAGCCC (position 315,857-315,874, 440bp amplicon). Specificity of the *Wolinella* spp. primers was tested on DNA extracts from *H. pylori* (ATCC 43504), *H. felis* (ATCC 49179), *H. bizzozeronii* (ATCC 700030), *"H. heilmannii" (*DNA from the stomach of an infected cat), *H. bilis* (ATCC 51630), *H. cinaedi* (ATCC 35683), *H. hepaticus* (ATCC 51450), *H. canis* (ATCC 51401), and *W. succinogenes*. DNA (2 µl) was heated to 95°C for 15 mins, followed by 35 cycles of: 95°C for 30s, 58°C for 30s, 72°C for 45s, and a final extension step of 72°C for 7 mins. DNA extracted from saliva samples of 20 dogs that were not examined by 16SrDNA sequencing were analyzed for the presence of *W. succinogenes* DNA by PCR. These animals were randomly sampled from the general practice and medicine caseload at Cornell University Hospital for Animals, and included healthy dogs as well as those undergoing clinical investigation for a wide variety of clinical signs.

Fluorescence in situ hybridization for *Helicobacteraceae* in saliva

Smears of saliva samples were also analyzed using fluorescence in situ hybridization (FISH) with *Helicobacteraceae* (HEL274/HEL717-Cy3) and eubacterial (EUB338-6FAM) probes, as previously described.[16,25]

RESULTS

Helicobacter PCR

The C97/C05 PCR was positive for *Helicobacter* spp. in the oral cavity DNA samples of 14/28 dogs, and gastric samples of 8/8 dogs.

Analysis of 16S rDNA clone libraries

- Oral cavity

A total of 57 clones from the oral cavities of 8 *Helicobacter* PCR positive dogs were sequenced (Table 7.1). Sequences with highest homology to *Wolinella spp.*, were the most prevalent (8/8 dogs) and abundant (52/57 clones, 91.2 %) in oral samples. Indeed, *Wolinella* was the only member of the *Helicobacteraceae* amplified from the dental plaque of 6/8 dogs. Sequences for *Helicobacter* spp. were detected in two dogs: dog 3 (healthy) had sequences with highest homology to "*H. heilmannii*" in dental plaque (1/7 clones), and dog 5 (vomiting) harbored sequences with highest homology to *H. felis* (3/6 clones) and "*H. heilmannii*" (1/6 clones) in saliva.

- Gastric mucosa

A total of 63 clones from the stomachs of 8 *Helicobacter* PCR positive dogs were sequenced (Table 7.1). The sequences had highest homology to *Helicobacter* spp. known to be present in the canine stomach: *H. felis* (n=6, 32/63 clones, 50.8%), "*H. heilmannii*" (n=5, 22/63 clones, 34.9%), and *H. bizzozeronii* (n=2, 9/63 clones, 14.3%) (Table 7.1). Of the 5 vomiting dogs,

Table 7.1: Members of the family Helicobacteriaceae identified by cloning and sequencing of 16S rDNA harvested from gastric biopsies, dental plaque and saliva of eight dogs originating from the patient population previously studied.[21]

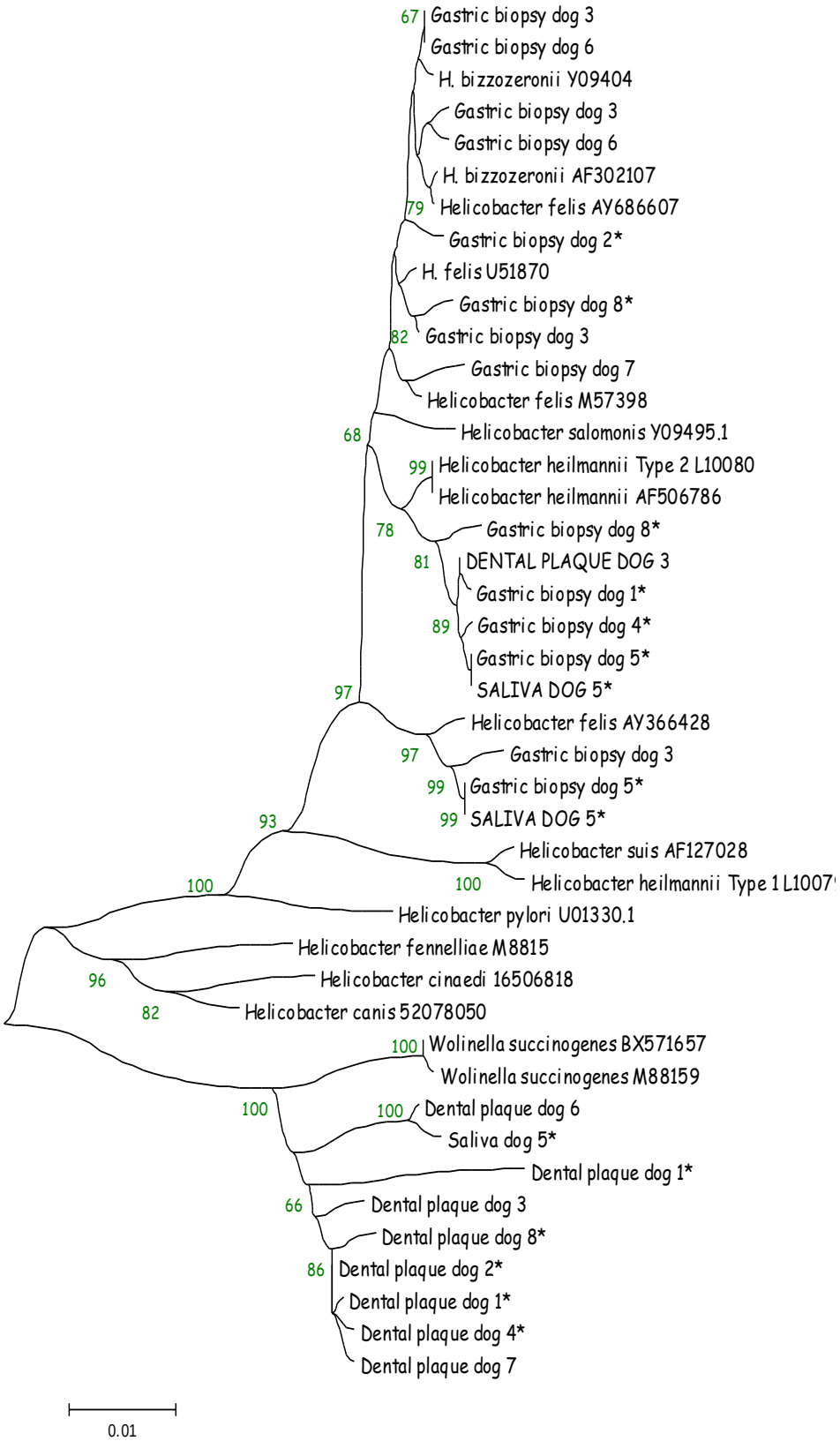
DOG	Vomiting V Healthy H	Time of sample post vomiting	Sampling site	*H. felis*	*H.heilmanni*	*H.bizzozeronii*	*Wollinella*	Closest matches according to NCBI and RDP II database searches	GenBank accession nos.	No. of clones	Similarity score (%)
1	V	>1 2h	Gastric		+			*"H. heilmannii"*	L10080 AF506786 AF506775	2 3 2	98.0, 98.3 98.2 - 99.0 97.0, 99.8
			Dental				+	*W. succinogenes*	M88159	6	96.7 - 98.1
2	V	48 h	Gastric	+				*H. felis*	M57398 U51870 AY686607	2 3 5	97.4, 98.5 98.3 - 99.0 97.5 - 99.3
			Dental				+	*W. succinogenes*	M88159	7	95.1 - 97.1
3	H	n/a	Gastric	+		+		*H. felis* *H. felis* *H. bizzozeronii*	U51870 AY366248 AF302107	2 3 2	96.0, 96.7 97.1 - 99.4 98.8, 99.6
			Dental		+		+	*"H. heilmannii"* *W. succinogenes*	AF506786 M88159	1 6	98.4 95.0 - 98.2
4	V	8h	Gastric		+			*"H. heilmannii"*	AF506786	7	96.3 - 98.6
			Dental				+	*W. succinogenes*	M88159	10	93.4-98.4
5	V	10 h	Gastric	+	+			*H. felis* *"H. heilmannii"*	AY366428 AF506786	4 3	96.9 96.8 - 98.6
			Saliva	+	+		+	*H. felis* *"H. heilmannii"* *W. succinogenes*	AY366428 AF506786 M88159	3 1 2	96.8-98.6 95.5, 96.6
6	H	n/a	Gastric	+		+		*H. felis* *H. bizzozeronii* *H. bizzozeronii*	U51871 AF302107 Y09404	3 3 4	99.8 - 99.4 98.7 - 99.3 98.5 - 99.3
			Dental				+	*W. succinogenes*	M88159	9	94.8 - 98.5
7	H	n/a	Gastric	+	+			*"H. heilmannii"* *H. felis* *H. felis*	AF506786 AY366428 M57398	2 1 3	95.2, 98.5 97.8 98.2, 99.2 99.0
			Dental				+	*W. succinogenes*	M88159	8	95.4-97.8
8	V	5h	Gastric	+	+			*H. felis* *H. felis* *"H. heilmannii"*	AY686607 U51870 AF506786	4 2 3	97.1-98.7 98.4-99.0 94.7-97.2
			Dental				+	*W. succinogenes*	M88159	4	95.1-96.8

sequences consistent with "*H. heilmannii*" were detected in 4 (20/40 clones, 50%) and *H. felis* in 3 (20/40 clones, 50%), with 2 dogs having a mixture of sequences. In the 3 healthy dogs, sequences with highest homology to *H. felis* were most prevalent (12/23 clones, 52.2%); followed by *H. bizzozeronii* (n=2, 9/23 clones, 39.1%), and "*H. heilmannii*" (n=1, 2/23 clones, 8.7%). A mix of sequences with highest homology to different *Helicobacter* spp. was present in the 3 healthy dogs: *H. bizzozeronii/H. felis* (n=2) and *H. felis*/"*H. heilmannii*" (n=1).

In the 2 dogs with concurrent oral and gastric *Helicobacter* spp., only one dog appeared to harbor highly similar sequences in both sites: dog 5 (vomiting) had sequences with highest homology to *H. felis* and "*H. heilmannii*" in both saliva and gastric mucosa, whereas dog 3 (healthy) had oral sequences with highest homology to "*H. heilmannii*" and gastric sequences with highest homology to *H. felis* and *H. bizzozeronii*.

The relationship of oral and gastric *Helicobacter* sequences in 16S rDNA clone libraries to each other and to reference sequences for a variety of *Helicobacteraceae* in GenBank was evaluated further by phylogenetic analysis (Figure 7.1). Sequences from the dental plaque of 8/8 dogs clustered with *Wollinella succinogenes* (bootstrap values 100%, nucleotide similarity scores 93.4-98.5%), and were clearly distinct from *Helicobacter* spp. The sequences from gastric mucosa clustered with reference sequences for *H. felis*, "*H. heilmannii*" type 2, *H. bizzozeronii* and *H. Salomonis*, with similarity scores 95.2-99.8%. Sequences from the gastric and oral cavity were clearly distinct (bootstrap value 97%) from "*H. heilmannii*" Type 1 (L10079: the predominant

Figure 7.1: Phylogenetic consensus tree showing the genetic relationship of 16S rRNA gene sequences amplified with C97/05 primers from gastric biopsy samples, dental plaque and saliva of 8 dogs (Sequences from vomiting dogs are asterisked). The numbers in boldface type at the nodes are the bootstrap percentages (1,000 replications; 65% cutoff). Vertical distance has no meaning. GenBank accession numbers are given after each reference isolate.


67
Gastric biopsy dog 3
Gastric biopsy dog 6
H. bizzozeronii Y09404
Gastric biopsy dog 3
Gastric biopsy dog 6
H. bizzozeronii AF302107
79
Helicobacter felis AY686607
Gastric biopsy dog 2*
H. felis U51870
Gastric biopsy dog 8*
82
Gastric biopsy dog 3
Gastric biopsy dog 7
Helicobacter felis M57398
68
Helicobacter salomonis Y09495.1
99
Helicobacter heilmannii Type 2 L10080
Helicobacter heilmannii AF506786
78
Gastric biopsy dog 8*
81
DENTAL PLAQUE DOG 3
Gastric biopsy dog 1*
89
Gastric biopsy dog 4*
Gastric biopsy dog 5*
SALIVA DOG 5*
97
Helicobacter felis AY366428
97
Gastric biopsy dog 3
99
Gastric biopsy dog 5*
99
SALIVA DOG 5*
93
Helicobacter suis AF127028
100
100
Helicobacter heilmannii Type 1 L1007
Helicobacter pylori U01330.1
Helicobacter fennelliae M8815
96
Helicobacter cinaedi 16506818
82
Helicobacter canis 52078050
100
Wolinella succinogenes BX571657
Wolinella succinogenes M88159
100
100
Dental plaque dog 6
Saliva dog 5*
Dental plaque dog 1*
66
Dental plaque dog 3
Dental plaque dog 8*
86
Dental plaque dog 2*
Dental plaque dog 1*
Dental plaque dog 4*
Dental plaque dog 7
0.01

subtype reported in human infections), *H. pylori,* and enteric *Helicobacter* spp. (*H. fenelliae, H. cinaedi, H. canis*).

In the two dogs with *Helicobacter* spp. sequences in their oral cavity, sequences from saliva and gastric mucosa of dog 5 clustered on the same branch, in groups of sequences with highest homology to *H. felis* and "*H. heilmannii*", suggesting colonization with similar strains at each site. In contrast the *Helicobacter* spp. sequences detected in the oral cavity and stomach of dog 3 segregated in 5 different clusters, suggesting colonization with different strains.

PCR for *Wolinella*

The results of 16S rDNA sequence analysis were unexpected, as we had anticipated the C97/C05 primer set was specific for the *Helicobacter* genus as reported by previous authors.[23,26] To investigate the prevalence of *Wolinella,* we self-designed PCR primers to *Wolinella* and checked their specificity using PCR against a panel of *Helicobacter* spp. and *W. succinogenes* DNA as the positive control (*H. pylori, "H. heilmannii," H. felis, H. cinaedi, H. bilis, H. bizzozeronii, H. hepaticus, H. pylori, H. canis, H. cinaedi*). We then performed PCR for *Wolinella* on DNA extracted from the saliva of the 20 dogs that had not been examined by 16S rDNA sequencing. Sixteen of 20 dogs were PCR positive for *Wolinella*, (Figure 7.2), giving a total of 24 of 28 (85.7%) dogs with *Wolinella* DNA present in their oral cavity.

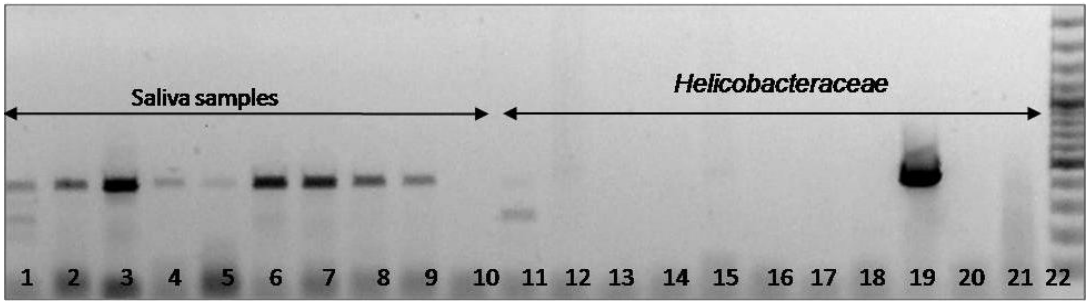


Figure 7.2: Agarose gel showing PCR products with *Wolinella*-specific primers performed on DNA extracted from the saliva samples of 10 pet dogs. Controls: 1- "*H. heilmannii*," 2-*H. felis*, 3- *H. cinaedi*, 4- *H.bilis,* 5- *H. bizzozeronii*, 6- *H. hepaticus*, 7- *H. pylori,* 8- *H. canis*, 9- *W. succinogenes.* 10 , 11 – negative controls, 12- 100bp ladder.

Fluorescence in situ hybridization (FISH)

The presence of *Wolinella* in the oral cavity was further examined using FISH for *Helicobacteraceae* on saliva smears (Figure 7.3). Though the probes are not *Wolinella*-specific, the characteristic lazy S-shape of the organism[27] was easily visualized, in contrast to the corkscrew morphology of *Helicobacter* spp.

DISCUSSION

Reports that the canine oral cavity is frequently positive for *Helicobacter* DNA (>70% of dogs[21]) raised the possibility that dogs may be an important zoonotic reservoir for increasingly recognized non-*pylori Helicobacter* gastritis in people.[4,10] To more clearly define the risk posed by contact with the oral cavity of dogs we sought to determine the specific *Helicobacter* spp. present in dental plaque and saliva. Our initial PCR screen with the widely used C97/C05 primer set indicated that *Helicobacter* DNA was present in the oral cavity in 14 out of 28 dogs (50%). However, 16S rDNA sequencing revealed that sequences with highest homology *to Wolinella* rather than *Helicobacter* spp*,* were the most prevalent *Helicobacteraceae* present. In marked contrast clone libraries of the gastric mucosa contained predominantly *H. felis,* and no detectable *Wolinella.* These results were unexpected, as we anticipated the C97/C05 would be specific for the *Helicobacter* genus, as reported by previous authors.[23, 26] Alignment of primer set C97 and C05 with the *W. succinogenes* genome sequence (www.wolinella.mpg.de/wolinella.html)[27] shows a 100% match at positions 1382756 and 1381565 respectively and yields a ~1200 bp

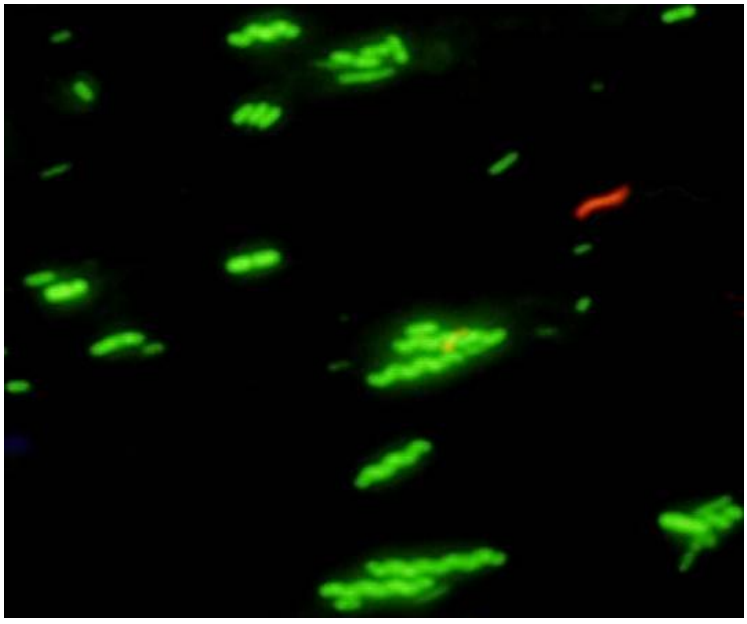


Figure 7.3: FISH image of a dog saliva smear, using *Helicobacter* red (Hel274/Hel717-Cy3) and eubacterial green (EUB-338-6FAM) probes. The characteristic 'S-shaped morphology of an organism presumed to be *Wolinella* is visible (red), surrounded by *non-Helicobacter* spiral bacteria (green).

DNA fragment that is indistinguishable in size from *Helicobacter* spp. Another commonly used *Helicobacter* 'genus-specific' primer pair C97/C98, (400 bp 16S rRNA amplicon) also closely matches the *W. succinogenes* genome, with just one mismatched base at the 5' end, (position 1382375). Thus primer set C97/C98 may also yield similar sized amplicons of *W. succinogenes* and *Helicobacter* spp and this may have produced false positive results for the presence of oral *Helicobacter* spp. DNA in the earlier work of Recordati et al[21] (reproducible here, unpublished data). We conclude therefore, that the 16S rRNA primers C97, C98, and C05 are *Helicobacteraceae*-specific and not *Helicobacter* genus specific as previously assumed.[23, 26] The *Wolinella*-specific PCR primers we designed should be helpful for detecting positives caused by *W. succinogenes* rather than *Helicobacter spp.*

Originally isolated from the rumen of cattle,[28] the genus *Wolinella* belongs to the epsilon subclass of the proteobacteria.[29,30] *W. succinogenes* has been previously isolated from the canine oral cavity[31] and was considered to be non-pathogenic until sequencing of the *W. succinogenes* genome[27] (www.wolinella.mpg.de) revealed homologous genes for virulence factors in *H. pylori* and *Campylobacter jejuni* (e.g. the type IV secretory pathway, adhesins, invasins and cytotoxins). This has lead researchers to question its non-pathogenic status,[27] and in fact a novel uncultured *Wolinella* spp. was identified in patients with squamous cell carcinoma of the esophagus[32] (*Candidatus W. africanus*)[33] in the upper digestive tract of asymptomatic Venezuelan and in the stomach of a horse with equine gastric ulcer syndrome.[34] New putative *Wolinella* species have been also identified in the stomach of a sea lion with gastritis.[35] Based on the adaptation of *Helicobacter*

species to different ecological niches of the gastrointestinal tract, Bohr et al. (2003) suggested that *Wolinella* species might be more adapted to colonize the squamous epithelium, since rumen and esophagus are both lined by this type of epithelium. The canine oral cavity, which is also lined by a squamous epithelium might represent another colonization site for *W. succinogenes*. Our findings support that *W. succinogenes* and *Helicobacter* spp. have distinct preferential colonization sites in dogs. The reason for the relative oral abundance of *W. succinogenes* compared to *Helicobacter* spp. is unclear, but may relate to subtleties in the oral microenvironment, such as pH, redox potential and nutrient availability. The growth of specific species may also be dependent on the presence or absence of other species in the microenvironment. For example, it has been shown that bacteria composing normal biofilm, such as *Streptococcus mutans* and *Prevotella intermedia*, can inhibit the growth of *H. pylori* strain.[36,37] Okuda et al (2000)[37] also discovered that the pathogenic periodontal bacteria *Porphyromonas* and *Fusobacterium nucleatum* can adhere to and trap *H. pylori*, and the incidence of oral *H. pylori* in humans may therefore vary according to oral health status. Whether or not the incidence of oral *Helicobacter* spp. in dogs is interlinked with oral health status remains to be determined.

Sequence based analysis of gastric *Helicobacter* spp. in the present study was broadly consistent with previous studies, with sequences with highest homology to "*H. heilmannii*", *H. felis* and *H. bizzozeronii* commonly identified. However, *Helicobacter* spp. with highest homology to *H. bizzozeronii* were less dominant than previous reports,[9,10,32,38] and were only detected in the gastric mucosa of the healthy dogs. In contrast "*H. heilmannii*"

was almost exclusively present in the vomiting dogs, which perhaps implies a more pathogenic role of these bacteria in dogs. The stomach of most of the dogs (5/8, 62%) appeared to be co-colonized with 2 *Helicobacter* species which is a higher rate of co-infection than previously reported (13.3%,[9] 16.6%[38]), but similar to the 55% reported by Van den Bulck et al (2005). *H. felis* and "*H. heilmannii"* sequences in the gastric mucosa and oral cavity of dog 5 clustered on the same phylogenetic branch and this may be related to recent vomiting (10 hours prior to sampling). Despite concordance in this dog, sequencing of oral *Helicobacter* spp. DNA does not reliably correlate with gastric spp, as demonstrated by dog 3 which harbored gastric sequences with highest homology to *H. felis* and *H. bizzozeronii* and oral sequences homologous to "*H. heilmannii*". Moreover, the presence of microbial DNA when determined solely by PCR is not indicative of colonization by viable bacteria.

In summary, the predominant member of the *Helicobacteraceae* inhabiting the canine oral cavity is *Wolinella*, which appears to preferentially colonize the oral squamous epithelium. Since only 8.8% of oral cavity clones were *Helicobacter* spp., the oral route may not be the predominant mode of transmission of gastric *Helicobacter* spp. between dogs. The low frequency of *Helicobacter* spp. clones, coupled with the absence of clustering with "*H. heilmannii*" Type 1, also implies that dogs likely pose little zoonotic risk for non-*pylori Helicobacter* infection in people. Larger studies encompassing a range of oral sampling sites and oral health status in dogs are required to confirm these initial findings and to investigate whether *W. succinogenes* has

a potential pathogenic role in canine oral disorders, as suggested by recent reports in humans and other mammals.

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CONCLUSIONS AND FUTURE DIRECTIONS

Owing to major technological advances in culture-independent molecular microbiology, we now recognize a pivotal role for bacteria in driving intestinal inflammation.[1,2] Each of the studies herein informs our understanding of host-bacterial relationships in gastrointestinal (GI) health and disease across species. In each of these studies the results translate to the clinical setting and inform disease management.

CHAPTER 2

This work reveals that independent of genetic susceptibility and injurious trigger, murine models of Crohn's ileitis develop severe Gram negative dysbiosis and proliferation of adherent invasive *E. coli* (AIEC). For the first time, we reveal that genetic susceptibility to IBD affects the threshold for dysbiosis in response to an external trigger, but is not a prerequisite for development of inflammation. Mucosal invasion by AIEC is shown to consistently accompany severe dysbiosis, and appears to be associated with a threshold of bacterial proliferation that in some way facilitates invasion. The impact of this work is to outline a hypothesis for chronic microbial-driven inflammation in ileal CD, after an acute, non-specific trigger of mucosal injury. Abrogated inflammation in CCR2 mice informs the clinical management of CD, and uncovers CCR2 blockade as a potential new therapeutic target.[3,4] Future work will be aimed at further unraveling the dialogue between the host inflammatory response and the microbiota. We aim to elucidate the specific components of the immune response and luminal conditions that upregulate Gram negative bacterial proliferation and facilitate AIEC invasion. Uncovering

these precise mechanisms will further inform disease pathogenesis and potentially uncover additional therapeutic targets.

CHAPTER 3

We are increasingly appreciating the role of mucosally invasive *E. coli* in Boxer dog granulomatous colitis (GC).[5,6] This work demonstrates that remission of GC hinges on successful eradication of mucosally invading *E. coli*, and that antimicrobial resistance is common amongst invading strains. Resistance to enrofloxacin is associated with a poor clinical outcome, and we speculate that this may be associated with the selection of resistant strains after short courses of empirical antibiotic therapy. Our findings directly impact disease management by highlighting the importance of definitive diagnosis and colon culture from the offset, rather than empirical management. The published clinical recommendation for practitioners is that antimicrobial therapy should always be guided by mucosal culture and antimicrobial susceptibility.

An important finding here was that all of the *E. coli* strains isolated were sensitive to amikacin. Unfortunately this drug is unsuitable for clinical application in GC, as the poor intracellular penetration of amikacin would be unlikely to eliminate intracellular *E. coli.* Future directions that are already underway involve development of amikacin conjugates with polymers that will enable intracellular drug penetration.[7-12] We also aim to perform further

antimicrobial susceptibility testing for multi-drug resistant strains, using combinations of antimicrobials. We hope to determine whether antimicrobials to which the *E. coli* is resistant demonstrate a synergistic effect when used in combination.

CHAPTER 4

The GWAS for Boxer dog GC has highlighted serious shortcomings in the Affymetrix platform and the MAGIC algorithm developed at Cornell that we were previously unaware of. We have halted further mapping of the candidate genes identified, as a result of being alerted to an unacceptable level of SNP genotype calling inaccuracy by the collaborating laboratory (Mezey Lab, Cornell). A recent study comparing the two platforms available for canine GWAS, Affymetrix and Illumina, also highlighted these problems, showing that as a consequence of LD variability from breed to breed, the tagging capacity and genome-wide coverage of both the Illumina 22K and Affymetrix 50K SNP arrays varied widely across breeds.[13] Higher density canine SNP arrays, such as the recently introduced 185K Illumina array, will improve power for individual genome-wide case–control association studies, and our plan is to repeat the GWAS using the Illumina platform for 24 affected and 24 control dogs. In this way, we hope to resolve the problems highlighted here, and clarify the GC-associated SNP associations.

Despite these problems, we have uncovered several potential candidate genes based on biologic function alone. The striking similarities between GC of Boxer dogs and CGD in people, as well as the existence of neutrophil dysfunction in 2 affected dogs clearly indicate that the NADPH

oxidase pathway deserves further investigation. This work as it stands has served to highlight striking parallels between Boxer dog GC and human CGD that were previously unappreciated by leading experts in both fields. As a result, evaluation of a specific role for AIEC in CGD is an important focus of future research that we will undertake using FISH and 16S rDNA pyrosequencing in people with CGD colitis.

CHAPTER 5.

This study demonstrates a unique form of PLE in the Yorkshire terrier (YT), associated with development of numerous, massively dilated intestinal crypts. Susceptibility of the YT for this unusual pathology has not been well documented previously.[14,15] We discovered high mortality rates and poor response to all therapies utilized, with long-term survival in only 3 of 14 dogs. FISH analysis showed no evidence of a bacterial association with crypt lesions, or obvious differences in the mucosa-associated flora compared to healthy control dogs (in our general experience, and compared to healthy dogs controls in Chapter 6). We conclude that YT suffer a severe and often fatal form of PLE that is consistently associated with apparently sterile, abscessated crypts. The absence of a bacterial association suggests that this may be a primary morphogenetic disorder with or without a secondary environmental trigger. The left shift neutrophilia, hyperfibrinogenemia and reactive thrombocytosis also suggest that this severe disorder has a marked systemic inflammatory component.

Our next step is to explore the underlying mechanisms for crypt abscessation. We aim to perform immunohistochemical evaluation of cell junction proteins, given the similarities that we have drawn with tufting enteropathy in people.[16,17] We will also stain lymphatic structures by immunohistochemistry, since we hypothesize that protein loss occurs predominantly via the crypt lesions rather than lymphatic dilation/lymphangiectasia.[18,19] We have successfully obtained a grant from the Royal College of Veterinary Surgeons (RCVS), UK, in order to perform this work. More thorough exclusion of viral agents should also be addressed, likely by performing immunohistochemical staining for parvoviral and coronaviral antigens. In view of the apparent breed specificity we plan to continue to prospectively identify affected cases in order to perform a genome wide association study for identification of candidate genes.[17]

Lastly, clinical implementation of adjunctive treatments using low molecular weight heparin, and perhaps in the future non-anticoagulant heparan sulfate should it become available.[20-22] The use of alternative immunosuppressive agents, e.g. cyclosporine, as outlined in the surviving cases, also warrants evaluation in a larger number of affected dogs.

CHAPTER 6

This is the first study to perform in-depth, culture independent prospective analysis of the canine intestinal microbiota alongside clinical phenotype (IBD vs healthy) and treatment response. Previous studies have been purely descriptive and make no attempt to translate their findings to the clinical setting in order to inform disease management.[23,24] This work uncovers

important differences in the duodenal microflora that may be useful in predicting treatment response in IBD affected dogs, involving predominantly the Bacteroidetes. An unexpected majority of dogs responded to dietary change alone here (62.5%). Even dogs with moderate to severe disease, and one with hypoalbuminemia achieved clinical remission on a hydrolyzed soy protein diet. We expect this finding to significantly impact canine IBD management, since it makes a good case for the treatment with hydrolyzed protein diets as the first step regardless of disease severity. The response to food was so rapid, that in stable cases even with severe disease, dietary trial is warranted. This opposes the approach currently widely adopted among practicing veterinarians and specialists, where animals with hypoalbuminemia are deemed to require aggressive immunosuppression. This is in part likely to be a consequence of 2 previous outcome studies of canine IBD where low albumin was identified as a negative prognostic indicator.[25,26] Wider clinical implementation of the standardized treatment protocol as adhered to in this work is justified by the outcome data.

Importantly, this is the first study to evaluate the canine intestinal microbiota of healthy pet dogs from a spectrum of breeds as controls, rather than laboratory-housed research animals. Comparison of 16S rDNA pyrosequencing data between these two populations will be of interest, since the majority of previous studies using control animals has relied largely upon laboratory Beagles. We speculate that there may be important differences in the intestinal microbiota between laboratory and domestic pet dogs due to environmental factors that raises their validity for use as a control population in studies of IBD. This study was expanded to 40 affected cases and 21 controls,

and future immediate directions will involve analysis of this larger cohort of animals in the same manner (Appendix 2).

CHAPTER 7

This study makes the important discovery that the oral cavity of dogs is infrequently inhabited by *Helicobacter* species. This serves to clarify a previous study, suggesting that dogs are a potential source of zoonosis for *non-H.pylori* infections.[27] The predominant member of the *Helicobacteraceae* inhabiting the canine oral cavity is in fact *Wolinella*, an apparently non-pathogenic organism. The low frequency of *Helicobacter* clones, coupled with the absence of clustering with "*H. heilmannii*" Type 1, implies that dogs actually pose minimal zoonotic risk for non-*pylori Helicobacter* infection in people. Larger studies encompassing a range of oral sampling sites and oral health status in dogs are required to confirm these initial findings. These data also reveals that PCR primers broadly targeting the *Helicobacter* genus (as opposed to specific species) must be carefully checked for specificity. The C97/05 primer set that previously published is not specific for *Helicobacters*, as previously assumed.[27,28]

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APPENDIX 1

Bioinformatics (Mezey Laboratory) Update for

Boxer GWAS, 12/8/10 (Chapter 4)

A mapping experiment was performed using a sample that included two distinct data sets. The first consisting of 17 boxers with colitis and 11 controls, which were phenotyped by the Simpson Laboratory at Cornell. The second was a set of 48 unaffected controls, obtained from a collaborator that were phenotyped by K Meurs, Washington State University, as part of a study of cardiac disease (ARVC) in Boxer dogs. The first sample was genotyped using the Affymetrix canine genotyping array V2.0 run in two separate batches processed at the Cornell Veterinary Medical College, where both cases and controls were present in both batches. The genotypes for this sample were called using BRLMM-P (Affymetrix) and the algorithm MAGIC version 3,[1] producing 17 affected and 11 control genotypes genome-wide, respectively. The second sample on the Affymetrix canine genotyping array V1.0, in a single batch with 48 genotypes provided by collaborators without allele identities.

We performed a GWAS using multiple tests to assess marker association with the case-control phenotype using PLINK including allelic, dominance, recessive and 2x3 table tests assessed using Fisher's exact method and with a Chi-Square test. We applied these tests while separately analyzing the first sample, as well as a combination of the first and second sample, where for the latter, genotypes were merged using allele frequencies. The first produced a large number of significant tests and a considerable early deviation from the null expectation when considered on a Quantile-Quantile

plot. There was a non-significant correlation between the first and second (combined) analysis, which we suspect reflected errors in combining the genotypes. We therefore did not consider the second, combined analysis further and focused our attention on the first sample analysis.

To assess whether population structure was driving the early deviation in the Quantile-Quantile plot, we used a Principal Component Analysis (PCA). MAGIC 3 and BRLMM-P datasets for these dogs were analyzed by PCA using the following filters: no missing data, sex chromosomes removed, and filtered for MAF 0.1; sex chromosomes removed only; and thinned genotypes (1 in every 10 used). Examination of the first three principal components in each analysis showed clustering, indicating cryptic population structure separating cases and controls. Given this structure, this likely explains the early deviation of the Quantile-Quantile plot and indicates the significant marker associations with disease may actually reflect an association with population structure, i.e. they are false positives for disease association.

In addition, an independent analysis of a set of Labrador retrievers for a Mast Cell Tumor (MCT) cancer phenotype, genotyped using the same Affymetrix platform by the same Veterinary College core facility, indicated an additional problem with the Affymetrix genotype calls. A Mendelian color trait was segregating in these Labradors, known to be a recessive allele at the MC1R locus, which was also independently genotyped in these dogs and showed a perfect association with the color phenotype, i.e. recessive homozygous with yellow, other genotypes with black/chocolate. This genotype was used as an internal control, since genotypes in sufficient linkage disequilibrium (LD) with this locus should show a perfect association with the

color phenotype.

Using BRLMM-P and MAGIC genotype calls of the Affymetrix array, there were a number of SNPs highly correlated with the causal locus. In addition, for a distinct set of Labradors that also included three of the same dogs used in the cancer study, an Illumina genotyping array was used to genotype the dogs, resulting in overlapping SNPs with the BRLMM-P and MAGIC genotypes that were highly correlated with the causal color locus. The Illumina genotypes in the MC1R color locus perfectly segregated with color when running case/control Fisher exact test association. In contrast, the MAGIC and Affymetrix BRLMM genotypes in MC1R did not segregate perfectly with color, even though some of the SNPs in the area were shared between all three calling methods. In addition, when comparing the three dogs for which we had both Illumina and Affymetrix arrays, the BRLMM-P and Illumina calls did not agree across the entire array at an unacceptably high level, where there were conflicting genotypes for about 40% of shared SNPs. These results indicate that the Affymetrix genotypes used in our study have a high enough error rate to prevent any observed significant associations from being interpreted as colitis associations, as opposed to genotyping errors.

Current status and future directions

Currently we have halted further mapping of the genes discussed herein, as a result of this update from the bioinformatics analyst of SNP genotype calling inaccuracy. A recent study comparing the two platforms available for canine GWAS, Affymetrix and Illumina, also highlighted these difficulties, showing that as a consequence of LD variability from breed to breed, the tagging capacity

and genome-wide coverage of both the Illumina 22K and Affymetrix 50K SNP arrays varied widely across breeds.[2] Higher density canine SNP arrays, such as the recently introduced 185K Illumina array, will improve power for individual genome-wide case–control association studies, and our plan is to repeat the GWAS using the Illumina platform for 24 affected and 24 control dogs. In this way, we hope to resolve the problems highlighted here, and clarify the GC-associated SNP associations.

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APPENDIX 2

Final study population for Chapter 6

IBD-AFFECTED DOG SIGNALMENTS (n=42)							
BREED	**Sex**	**Age**	**Wt**	**BREED**	**Sex**	**Age**	**Wt**
Pomeranian	MN	11.00	4.40	Am. Bulldog	MN	2.00	41.50
Labrador	FN	4.00	26.80	Fbulldog	ME	5.00	13.20
JRT	MN	9.08	6.30	GSD	FN	6.00	32,4
GSD	FN	4.50	28.40	Gt dane	MN	2.00	51.60
SCWT	FN	14.50	9.00	Xbreed	FN	13.56	7.30
WHWT	MN	4.00	8.80	Bulldog	FN	9.80	30.90
Sheltie	MN	11.00	10.10	Malamute	FN	12.00	29.00
Boxer	MN	5.50	31.70	Labrador	MN	10.50	27.20
Xbreed	MN	10.00	27.20	Labrador	MN	12.00	28.10
Xbreed	FN	1.50	20.80	Irish Setter	ME	1.00	25.80
Bassett	FN	1.40	14.50	Boxer	ME	1.00	27.90
Yorkie	MN	7.00	2.50	Xbreed	MN	14.00	4.00
Boxer	FN	5.00	24.50	Labrador	FN	9.72	27.50
Schnauzer	FN	8.00	5.60	Pomeranian	MN	13.00	4.40
Yorkie	FN	3.08	2.40	Yorkie	FN	4.00	3.00
Eskimo dog	FN	12.50	7.50	BichonFrise	FN	13.00	7.90
SCWT	MN	1.00	12.40	Xbreed	FN	10.00	20.40
Xpug	FN	10.32	7.10	St Bernard	FN	2.00	43.50
Labrador	ME	4.00	24.80	Xbreed	MN	2.50	9.40
Am. Cocker	FN	2.00	9.60	Boxer	MN	8.00	25.90
Labrador	MN	10.00	45.00	WHWT	MN	2.00	9.00

HEALTHY DOGS (n=21)	
Labrador	GSD
Labrador	Xbreed
Labrador	Xbreed
Labrador	Greyhound
Labrador	Xbreed
Labrador	Xbreed
Labrador	Cocker Spaniel
Labrador	Xbreed
Labrador	Ridgeback
Bulldog X	Boxer
Xbreed	

APPENDIX 3

Abstract Presentations and Awards

CHAPTER 2: presented in 2 stages as oral abstracts at Digestive Diseases Week (DDW) 2009 and 2010.

i. M Craven, C Egan, B Dogan, E Denkers, SE Dowd, S McDonough, E Scherl, KW Simpson. Acute Intestinal Inflammation In Mice Decreases Microbial Diversity And Triggers Proliferation Of Adherent Invasive *E. Coli*.

Awarded best student oral abstract at DDW 2009, by the American Gastroenterology Association.

ii. M Craven, C Egan, B Dogan, E Denkers, SE Dowd, S McDonough, E Scherl, KW Simpson. CCR2 Deletion Ameloriates Ileal Inflammation, Limits Dysbiosis And Prevents Invasion By Adherent And Invasive *E. Coli* (AIEC) In *T. Gondii*-Infected Mice.

Awarded best student oral abstract at DDW 2010, by the American Gastroenterology Association.

CHAPTER 3: presented as an oral abstract at the Forum of the American Congress of Veterinary Internal Medicine (ACVIM), 2009.

M Craven, B Dogan, A Schukken, M Volkman, A Chandler, PL Mcdonough, KW Simpson. *E. Coli* Associated With Granulomatous Colitis Of Boxer Dogs Frequently Manifest Resistance To Antibiotics.

CHAPTER 4: presented as an oral research report at the Forum of the ACVIM, 2010 and poster abstract at DDW 2010.

M Craven, GM Acland, JG Mezey, AR Boyko, W Wang, K Meurs, SP McDonough, KW Simpson. Genome-Wide Analysis Of Granulomatous Colitis In The Boxer Dog.

CHAPTER 5: presented as a poster abstract at the Forum of the ACVIM 2009.

> M Craven, GE Duhamel, NB Sutter, KW Simpson. Absence Of A Bacterial Association In Yorkshire Terriers With PLE And Cystic Intestinal Crypts.

CHAPTER 6: presented as an oral abstract at the Forum of the ACVIM 2009.

> M Craven, SE Dowd, S McDonough, KW Simpson. High Throughput Pyrosequencing Reveals Reduced Bacterial Diversity In The Duodenal Mucosa Of Dogs With IBD.

Awarded best oral abstract in the gastroenterology section by the Comparative Gastroenterology Society.

CHAPTER 7: presented as a poster abstract at the forum of the ACVIM 2010.

> M Craven, C Recordati, V Gualdi, G Pengo, M Luini2, E Scanziani, KW Simpson. *Wolinella Succinogenes* And Not *Helicobacter* Spp. Are The Predominant Helicobacteraceae In The Canine Oral Cavity.

APPENDIX 4

Additional PhD Activities

1. Investigation of mucosal cytokines in feline IBD and alimentary lymphoma. Oral abstract presentation at the Forum of the ACVIM, 2010

Mucosal Cytokine Profiling Reveals Il-6 Up-Regulation in Feline IBD and Alimentary Lymphoma.

M Craven, RE Goldstein, RA Chiotti, A Greiter-Wilke, S McDonough, KW Simpson.

Chronic enteropathies are widely thought to result from dysfunction of innate and/or adaptive mucosal responses to intestinal antigens in a susceptible individual. Lymphoproliferative disease is a well-recognised sequela to chronic intestinal inflammation in people, and though this has not been critically examined in dogs and cats, the increasing prevalence of intestinal T cell lymphoma in cats in particular could be preceded by chronic inflammation. The mucosal cytokine milieu is thought to play a pivotal role in neoplastic transformation in people, with IL6, TGFb and TNFa implicated. We hypothesize that intestinal cytokine responses differ in healthy cats versus those with IBD and lymphoma and that cytokine profiling might be a useful adjunct to disease diagnosis. Endoscopic duodenal biopsies were obtained from 16 cats referred for evaluation of chronic gastrointestinal (GI) signs. Biopsies from 8 healthy DSH cats were included as controls. Tissue was collected into RNA later (cytokines), and formalin (histology and T cell receptor gamma, TCRG, gene analysis). Cytokine mRNA (IL-1a, IL-1b, IL-4, IL-6, IL-8, IL-10, IL-12p40, TGFb, TNFa, IFNg) was measured by probe-based real-time RT-PCR, normalised to GAPDH, and expressed as CT. TCRG gene clonality was evaluated by PCR amplification (CDR3) and PAGE on native and denatured PCR products, with samples classified as clonal (C), oligoclonal (OC), polyclonal (PC) and pseudoclonal (PS). The histological diagnosis was lymphoma in 6 cases (4/6 confirmed as T cell by immunophenotyping), IBD in 7, and equivocal for IBD/lymphoma in 3. TCRG gene analysis in lymphoma cases was OC in 4/6, C 1/6, PC 1/6; in IBD cases was OC in 2/7, C 1/7, PC 3/7, PS 1/7; and in equivocal cases was OC in 2/3, and PC 1/3. Cytokine profiles for IL-1a, IL-1b, IL-4, IL-8, IL-10, IL-12p40, TGFb, TNFa and IFNg analyzed by histologic diagnosis were similar in lymphoma, IBD, and healthy cats. Amplification of IL-6 was significantly associated with the presence of GI disease ($p<0.001$, Fisher's exact). IL-6 was quantifiable in only 2/8 healthy cats, (mean CT 13.15) versus 6/7 IBD (mean CT 3.34), 6/6 lymphoma (mean CT 2.83) and 3/3 equivocal cases (mean CT _0.32). There was no difference in IL-6 between IBD and lymphoma. When IBD and lymphoma were analyzed according to TCRG gene clonality there was no significant difference in IL-6 levels between OC/C and PC/PS. We conclude that mucosal IL-6 is a potentially useful adjunctive diagnostic marker for feline IBD and lymphoma. The IL-6 dominated intestinal cytokine milieu in feline enteropathies suggests that IL-6 mediated resistance to apoptosis may be associated with inflammation and neoplastic transformation, as has been recognized in people.

2. Other research projects contributed to:

   i. Mansfield CS, James FE, **Craven M**, et al. Remission of Histiocytic Ulcerative Colitis in Boxer Dogs Correlates with Eradication of Invasive Intramucosal *Escherichia Coli.*
   J Vet Intern Med 2009;23:964-969.

   ii. Jergens AE, Pressel M, Crandell J, **Craven M,** et al. Fluorescence in Situ Hybridization Confirms Clearance of Visible *Helicobacter* Spp. Associated with Gastritis in Dogs and Cats.
   J Vet Intern Med 2009;23:16-23.

   iii. Recordati C, Gualdi V, **Craven M,** et al. Spatial Distribution of *Helicobacter* Spp. in the Gastrointestinal Tract of Dogs. *Helicobacter* 2009;14:180-191.

   iv. Egan C, **Craven M**, Leng J, et al. CCR2-Dependent Intraepithelial Lymphocytes Mediate Inflammatory Gut Pathology during *Toxoplasma Gondii* Infection.
   Mucosal Immunol 2009;2:527-535.

   v. Sheldon M, Rycroft A, Dogan B, **Craven M**, Bromfield J, Roberts M, Price S, Gilbert R, Simpson KW. Specific Strains of *Escherichia coli* Are Pathogenic for the Endometrium of Cattle and Cause Pelvic Inflammatory Disease in Cattle and Mice. PLoS ONE 5(2):1371.

   vi. Kornreich B, **Craven M**, McDonough SP, Nydama DV, Simpson KW. Fluorescent in situ hybridization for the identification of bacterial species in archival heart valve sections of canine bacterial endocarditis (submitted).

   vii. Fluorescence in situ hybridization studies of:
   - Pancreatitis in cats
   - Cholangiohepatitis in cats
   - Trichinella infections in mice
   - TLR2 and TLR4 knockout experiments in mice

   viii. Real-time PCR for *E. coli* uidA copy # on ileal biopsies from Crohn's Disease patients.