DISSERTATION

COMPREHENSIVE INVESTIGATION OF CHRONIC ENTEROPATHY IN DOGS THROUGH A PROSPECTIVE CLINICAL TRIAL, IMMUNOASSAYS, AND RNA-SEQUENCING

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ABSTRACT

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Chronic enteropathy is a common condition in dogs causing recurrent or persistent gastrointestinal clinical signs. Pathogenesis is thought to involve intestinal mucosal inflammatory infiltrates, but histopathological evaluation does not predict treatment response, inform prognosis, or correlate with clinical remission. Many dogs may improve clinically with dietary intervention, but between 15 to 40% of dogs are refractory to all therapies. This negatively impacts quality of life for dogs and their families and can lead to euthanasia. Better understanding of the cellular and molecular differences between CE and health is necessary to improve outcomes for these dogs, and to enable use of the dog as a translational model for study of inflammatory intestinal conditions across species. The goal of this work was to critically evaluate the pathogenesis of CE in dogs through use of in vitro assays, a prospective clinical trial, and next-generation sequencing based approaches.

Preliminary studies have highlighted an important role for intestinal bile acids in the pathogenesis of canine and human chronic enteropathies. Fecal bile acid populations differ between healthy dogs and dogs with CE. However, there has been little work to evaluate potential consequences of these metabolic shifts in dogs. We therefore investigated potential immunomodulatory roles of primary and secondary bile acids through in vitro experiments with canine macrophages. Both the primary bile acid cholic acid (CA) and the secondary bile acid lithocholic acid (LCA) influenced LPS-induced cytokine production via canine monocyte-derived macrophages similarly, with suppression of TNF-α secretion and enhancement of IL-10 secretion. Neither BA altered the expression of the BA receptor TGR5. Transcriptomic analysis

revealed that CA activated inflammatory signaling pathways in macrophages involving type II interferon signaling and the aryl hydrocarbon receptor, whereas LCA activated pathways related to nitric oxide signaling and cell cycle regulation. Thus, we concluded that both primary and secondary BAs are active modulators of macrophage responses in dogs, with differential and shared effects evident with sequencing analysis.

Diet is the most effective management strategy for dogs with CE, enabling two-thirds of patients to achieve clinical remission from their disease. Various dietary strategies may be beneficial. Nutritional formulae sourcing protein from amino acids have been used for the induction of remission in human Crohn's disease patients for decades. We conducted a prospective clinical trial involving exclusive feeding of the first diet sourcing protein from individual amino acids to 23 client-owned dogs with CE to determine its ability to induce clinical remission and begin to tease apart mechanisms of action. After 2 weeks of EL, 68% of dogs consuming the diet were classified as responders. At the conclusion of the 8 week feeding trial, 16/23 dogs (70%) were considered clinical responders. Feeding EL caused shifts in fecal bacterial communities, which differed between responders and non-responders, suggesting that diet's ability to modulate gut bacterial populations may predict its efficacy. Serum biomarker concentrations were unchanged throughout the study apart from serum alkaline phosphatase activity. Results of this study indicate that an amino acid based diet is another option to treat dogs with CE and implicates the intestinal microbiota in achievement of remission in these patients.

Most studies comparing healthy and CE dogs completed to date have been limited in scope, evaluating individual or a small collection of biomarkers or cell types. This has hampered advancement of the understanding of CE pathogenesis in dogs. Ultimately, this results in generic treatment strategies for dogs and leaves a substantial proportion unable to achieve clinical remission from their disease. To this end, we applied next-generation transcriptomic sequencing to mRNA from duodenal biopsies from CE dogs and healthy beagle dogs. Results

of this analysis highlighted important roles for epithelial cell gene signatures in differentiating CE tissues from healthy ones. Commonly implicated cytokines like TNF-α, IL-12, or IL-10 were not differentially expressed, but pathway analysis highlighted a potential role for upregulation of anti-viral pathways in CE dogs. This preliminary study underscores the power of RNA sequencing to provide a broad overview of cellular activities in tissues of interest, and question widely accepted theories regarding dysfunction present in the gut of dogs with CE.

Single-cell RNA sequencing offers a high-resolution molecular technique enabling characterization of gene expression on an individual cell basis. This approach overcomes traditional barriers to disease investigation (e.g., species-specific reagents) and allows for definition of cell subtypes within heterogeneous samples. We thus employed single-cell RNA sequencing to catalog and compare the diversity of cells present in duodenal mucosal endoscopic biopsies from 3 healthy dogs and 4 dogs with CE. We identified populations of epithelial cells, T cells, myeloid cells, and plasma cells, with contributions from both the healthy and CIE samples. Neutrophils from CE samples exhibited a more inflammatory transcriptional program. T cells were broadly divided into non-resident and tissue resident subtypes, though minimal transcriptomic differences were appreciated within this class of cells. One subset of epithelial cells from CE dogs showed differential expression of a gene encoding a 2-pore potassium channel (KCNK16). Our results reveal a previously unappreciated cellular heterogeneity in canine duodenal mucosa and provides insights into molecular mechanisms underlying CE in dogs. The cell type gene signatures determined through this work will enable better understand the subtleties of canine intestinal physiology to allow more accessible interrogation of cellular activities in health and disease.

The results of the studies described add further nuance and detail to understanding of the pathogenesis and management of canine CE. We have documented the power of transcriptomic analysis for differentiation of intestinal mucosal molecular programs in health and

CE. Further investigation into intestinal bile acids, duodenal mucosal T cell subtypes and neutrophils, and intestinal epithelial cell activities are indicated.

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DEDICATION

This dissertation is dedicated to the memory of Ruth Haglund Manchester, my nana. Pursuit of higher education was not a possibility afforded to her, yet she is one of the smartest and kindest people I have ever known.

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1.1 Overview of Canine Chronic Enteropathy

Terminology and Prevalence

Chronic enteropathy (CE) is a common but poorly understood condition in dogs involving recurrent or persistent gastrointestinal (GI) clinical signs[1,2,3]. Diagnosis of CE requires that dogs meeting certain criteria. Firstly, they must display clinical signs compatible with GI tract dysfunction, such as diarrhea, vomiting, reduced appetite, unexplained weight loss, as well as abdominal pain, bloating, regurgitation, and/or flatulence. To be considered chronic, signs must last for longer than 3 weeks. Many dogs present with signs that wax and wane recurrently over months to years. Given that extra-GI problems may secondarily cause clinical signs compatible with CE, a workup must be completed to rule out hepatic, renal, metabolic, and endocrine conditions. Abdominal imaging helps to rule out structural drivers of GI clinical signs. Once extra-GI causes are ruled out, the goal is to identify specific primary GI causes distinct from CE. Histopathologic analysis of intestinal biopsy samples is a controversial aspect of CE diagnosis. Documentation of a range of types and severities of inflammatory mucosal infiltrates, without evidence of neoplasia or infectious agents, may further solidify the diagnosis of CE. The term chronic inflammatory enteropathy (CIE) refers to dogs meeting the above criteria who have had histopathologic evaluation of intestinal mucosal biopsies[4]. All in all, CE is a non-specific syndrome that encompasses a wide array of patients.

Acute and chronic GI disturbances, manifesting as abnormally loose or frequent stools (diarrhea), vomiting, and poor appetite, occur frequently in pet dogs. Evaluation of health insurance claims from dogs within the United States indicates that diarrhea and vomiting were the third and fourth most common reasons for presentation for veterinary evaluation[5]. Another large insurance provider cited “stomach issues” as the top reason for claims in 2019; these accounted for ~800,000 claims[6]. In England, evidence suggests that digestive disorders account

for 30% of primary care veterinary visits; this was the second most common organ system affected following dermatologic disorders[7]. Determining what proportion of dogs present for chronic rather than acute GI issues is difficult to discern. Retrospective investigations of dogs seen at referral practices for chronic enteropathies are limited. Available data from England and Italy suggests 1 to 2% of these dogs suffer from chronic GI conditions[8,9]. Prevalence data specific to referral clinics in other parts of the world is lacking[10], and though it seems as if the prevalence of CE has grown over time, this has not been proven.

Of the entire population of dogs seen by a veterinarian for chronic GI clinical signs, CE appears to be the most common etiology. Among 136 dogs with chronic diarrhea, Volkmann et. al. found that 71% were ultimately diagnosed with CE (histopathologic evaluation of intestinal biopsies performed in 34/97 dogs); infectious enteritis (i.e., parasitism or protothecosis) was the second most common diagnosis, identified as the cause of diarrhea in 13% of dogs[11]. Another study limited to dogs undergoing endoscopy for their CE clinical signs reported that 191 of 195 (97.9%) of cases were diagnosed with CIE[12]. This is in line with our experience at Colorado State University's Veterinary Teaching Hospital (CSU-VTH).

Canine CE is an umbrella diagnosis, encompassing a mix of patients experiencing persistent GI clinical signs. Current classification schemes for CE include retrospective assignment based on response to treatment; categories include diet or food-responsive, antibiotic-responsive, and immunosuppressant- (or steroid-) responsive enteropathy[3]. If a dog's clinical signs dramatically improve during the treatment trial, they are assigned that CE subtype. Lack of pathognomonic clinical manifestations or specific biomarkers or leaves treatment trials as the only means of arriving at these "diagnoses". Typically, a dedicated 10 to 14 day feeding trial with a specific diet is enacted to determine if the dog has diet-responsive disease. This approach is imperfect as it is impossible to predict which dietary strategy will most benefit an individual patient, and so dogs may be inappropriately classified as diet non-responsive because they were not fed the ideal diet. The most common antimicrobials administered to

manage CE with dogs are metronidazole, tylosin and oxytetracycline[11,13]. Dogs whose CE clinical signs improve with antibiotic administration are deemed antibiotic-responsive. Remission tends to be transient, with most dogs have recurrence of their clinical signs when antibiotics are discontinued[14]. This suggests that tylosin and metronidazole temporary suppress clinical signs rather than treating or curing enteropathogenic infection. Immunosuppressants are often implemented after diet and antibiotics given high likelihood of side effects[2,15]. Commonly utilized drugs include prednisone, cyclosporine, azathioprine, and chlorambucil[3,16, 17,18]. Budesonide is a glucocorticoid option that may have fewer systemic side effects due to its high first-pass hepatic metabolism[15,19]. Dogs that fail all treatment strategies are considered to have non-responsive enteropathy. All in all, these categories are of limited practical utility. The time required to complete these sequential trials results in unacceptable morbidity and cost to the pet owner. Ultimately, many dogs require multimodal therapies to achieve clinical remission. More sophisticated means of subdividing the heterogeneous collection of dogs with CE have not been accomplished to date; better understanding of CE would be required to allow for more streamlined, individualized patient management.

The term CE (or CIE if intestinal biopsies have been evaluated) is currently preferred over inflammatory bowel disease (IBD) to describe dogs with chronic non-specific enteropathy. This helps to provide some separation between conditions seen in dogs and in humans[3]. This is important because most dogs with CE do not display phenotypes analogous with the main forms of human IBD, Crohn's disease (CD) and ulcerative colitis (UC)[20]. These diseases have a much more aggressive clinical course and, in the case of CD, often require surgical management[21]. Washabau et. al.'s previously proposed diagnostic criteria for IBD in dogs involved exclusion of all dogs with diet- and antibiotic-responsive CE[22]. Within that "all-encompassing definition", dogs with IBD were limited to the immunosuppressant-responsive enteropathy category described by others[3,23]. Given the absence of identifiable characteristics distinguishing dogs responding to different management strategies[17,24,25], the author prefers a

definition of CE more in keeping with Dandrieux et. al. 2016. For the purposes of this document, CE refers to dogs with chronic GI clinical signs for which specific causes have been ruled out, with normal serum albumin concentrations, with or without lymphoplasmacytic to eosinophilic intestinal mucosal infiltrates (if evaluated), regardless of what treatment they respond to.

A few conditions may present identically to CE in dogs but are clearly different diseases. These patients must be identified and excluded from the heterogeneous CE population. Diseases that may masquerade as non-specific CE include infectious enteridities (e.g., histoplasmosis, protothecosis, *E. coli*-associated granulomatous colitis, schistosomiasis) and GI neoplasia (e.g., lymphoma)[26,27,28,29,30,31]. Protein losing enteropathy (PLE) is another cause for chronic GI clinical signs which is sometimes included under the CE umbrella[32]. Some perceive PLE to be a more severe form of CE, but a substantial body of evidence points to this being a distinct entity[33,34,35]. Dogs with PLE show low serum albumin or panhypoproteinemia, often combined with hypocholesterolemia and thrombocytosis[32,36]. Third-spacing of fluid, resulting in ascites or subcutaneous edema, often develops due to inadequate oncotic pressure. They suffer from distinct disease complications including thromboembolic events and micronutrients deficiencies (i.e., tryptophan, vitamin D3)[37,38,39]. Prognosis for PLE dogs is worse than that for dogs with CE and normal serum albumin concentrations[11,17,40]. This dissertation will focus on normalbuminemic CE dogs and so does not pertain to PLE.

Current Clinical Approach to Chronic Enteropathy in Dogs

The clinical approach to dogs with chronic GI signs begins with a detailed clinical history. Information gleaned may help localize disease to the upper GI tract in dogs with regurgitation, dysphagia, odynophagia and/or vomiting[40]. Diarrhea can be seen with disorders of the small and large intestine. An exact definition of diarrhea in canine patients is lacking, but most studies rely on pictorial stool scoring charts, with stools ranging from formed and dry (score 1) to watery and without shape (score 7). Workup is focused on investigation of small intestinal diseases in

dogs with loose, voluminous stools and/or weight loss. Diseases localized to the large intestine are prioritized in dogs with diarrhea characterized by small, frequent bowel movements, mucus, hematochezia, and/or tenesmus without weight loss or vomiting[2]. In most cases, these anatomical distinctions are not overly helpful, and a general approach is implemented. Beyond characterization of clinical signs, responses to prior interventions are key components of the clinical history which can lead to reordering of the differential diagnosis list. Once history has been delineated, workup entails an iterative process to rule out specific causes for the clinical signs present. Secondary or extra-GI cause for signs including pancreatic (e.g., exocrine pancreatic insufficiency, EPI, chronic pancreatitis), endocrine (e.g., hypoadrenocorticism, hypothyroidism), hepatic, and renal conditions should be excluded first. Exclusion of these causes is accomplished with a minimum database (complete blood count, chemistry panel, urinalysis) and endocrine testing (ACTH stimulation test, thyroid hormone testing). Mild biochemical abnormalities are not uncommon with CE dogs. For example, mild non-regenerative anemia has been identified in 12 to 18% of CE dogs[40,41]. In most CE dogs, the minimum database is unremarkable, and a primary GI cause for CE clinical signs is prioritized. Next, readily treatable causes of CE signs such as intestinal parasitism are screened for with fecal parasite testing and/or treated empirically with broad-spectrum deworming. At this point, practitioners have several options in terms of patient management, including treatment trials and further diagnostic workup.

For practitioners implementing a treatment trial approach, a dedicated feeding trial of a new diet is a common next step. Guidelines suggest a gradual transition from the current to the selected diet over a period of 4 to 7 days, followed by at least 2 weeks of exclusive feeding (meaning the dog gets all its calories from the indicated diet) with monitoring of clinical signs (Mueller 2018). Many studies have shown that if diet change is going to help reduce or resolve clinical signs, it will do so within the first 7 to 14 days of feeding[17,42,43]. Multiple different diet trials should be implemented before confidently excluding diet-responsive disease[44]. For more on the

role of diet in CE management, see section 1.3. Dogs continuing to have clinical signs despite diet trials may be prescribed an antibiotic such as metronidazole, tylosin or oxytetracycline. Dogs with antibiotic-responsive CE tend to younger and larger breed, with German shepherds overrepresented within this category[45]. Immunosuppression is usually the third strategy implemented. Commonly utilized drugs included prednisone, budesonide, cyclosporine, chlorambucil, and mycophenolate. Dogs responding to medical strategies may be kept on the drugs indefinitely or have the dose tapered to the lowest effective dose. This trial-and-error approach is laborious and non-specific, and dogs may have worsening of their disease when unhelpful treatments are implemented.

Disease severity in dogs with CE may be assessed using a clinical scoring index. The first to be developed was the canine inflammatory bowel disease clinical activity index (CIBDAI) [46]. This consists of a series of questions regarding frequency and severity of GI signs and ranges from 0 (normal) to 18 (severe disease). Allenspach and colleagues updated the index in 2007, adding in parameters regarding serum albumin concentration and fluid accumulations (e.g., edema, ascites), as well as pruritis[17]; this is the canine chronic enteropathy clinical activity index (CCECAI). Scores range from 0 (normal) to 27 (severely affected). The main utility of the CCECAI over the CIBDAI is the inclusion of attributes related to PLE. *How do these clinical indices help guide management?* A study of 70 dogs with CE signs identified a significant association between CIBDAI score and refractoriness to treatment or euthanasia, but 15 of 70 dogs in this study were hypoalbuminemic (serum albumin < 2.0 g/dL)[17]. Nevertheless, when PLE dogs were excluded, the odds ratio for a poor outcome remained significant (OR 1.5, 95% confidence interval 1.1-2.1; P = .02). Some studies have suggested that dogs with higher clinical activity scores are less likely to respond clinically to diet[11,17,42]. Older guidelines recommended a “top down” treatment approach, involving concurrent implementation of dietary, antibiotic, and immunosuppressive interventions[2,47]. However, dogs with moderate to severe clinical disease may achieve clinical remission with diet alone[24,48,49], and dietary trials can be

implemented with essentially no risk. Therefore, CIBDAI or CCECAI values do not foretell treatment response category and are best used to establish a baseline to be able monitor response to treatment trials.

Prognosis

Chronic enteropathy typically results in morbidity rather than mortality, with quality of life for dogs and their families being negatively impacted by diarrhea, lack of interest in food, vomiting, and so on[50]. However, in some cases, dogs may have such severe clinical signs that euthanasia is pursued due to perceived poor quality of life[17,40,51]. Estimating prognosis in dogs with CE at the time of presentation is challenging. The best long-term prognostic indicator identified thus far is a positive clinical response therapeutic intervention; specifically, dogs responding to dietary intervention have the best long-term outcomes in terms of abatement of clinical signs with minimal relapses[23,52]. Long-term outcomes of dogs within the antibiotic- or immunosuppressant-responsive categories are poor, with frequent relapses[23]. One study of 80 dogs with CE that was not responsive to diet alone found that owner assessed quality of life at follow-up visits was the only factor predictive of a negative outcome. Of note, no associations were identified between disease severity or localization (i.e., upper, SI, large intestine) and outcome[40]. Many other studies have mined the clinical data for predictors of poor outcomes; factors identified in more than 1 study include older age, higher disease severity scores, and predominantly SI disease[17,23,40,47]. The only clinicopathologic indicator that has been associated with a worse prognostic in normalbuminemic CE dogs is severe hypocobalaminemia (serum B12 concentration < 200 pg/mL)[11,17]. Hypovitaminosis D has been identified as a negative prognostic indicator, but it is strongly and significantly correlated with serum albumin concentrations[37,53,54]. Therefore, the relevance of these biomarkers as prognostic indicators for CE dogs without hypoalbuminemia is uncertain. In general, assigning an accurate prognosis to

CE dogs is challenging using available diagnostic tools. Improved targeting of therapies to individual patients could therefore have a massive impact on morbidity among dogs with CE.

Translational Relevance

Dogs are excellent large animal models for translational study of chronic intestinal conditions. The domestic dog has evolved alongside humans for at least 10,000 years[55], and pet dogs are exposed to many of the same environmental factors (e.g., diet, antibiotics, infectious agents) that contribute to disease in humans[56,57,58]. Dogs and humans are omnivorous species, with broad overlap in GI physiology[59,60] and >17,000 shared orthologous genes[61]. Ongoing progress annotating the canine genome has advanced the potential for canine data to contribute translational understanding of GI physiology and pathophysiology[62]. While pet dogs' activities and food intake are not as readily controlled as can be achieved with mice or rats, dogs are customarily fed a single diet, reducing the contribution of that influential variable.

Unsurprisingly, veterinarians and researchers have extrapolated findings research in humans to canine patients and conditions, with varying success. The most straightforward comparisons are found in infectious diseases with pathogens causing disease in both species. *E. coli*-associated granulomatous colitis, predominantly affecting Boxers and French bulldogs, shares many features with Whipple's disease[63]. Genetically similar *E. coli*, able to replicate and persist in mammalian cells, have been isolated from 36% of ileal biopsies from Crohn's disease patients[64]. Relating non-infectious GI diseases across species is less straightforward. A Celiac disease-like condition has been described in Irish Setter dogs[65,66], whereby a T-cell driven reaction is mounted against gluten, one of the proteins in wheat. This breed-associated nature of this condition parallels the situation in people where genetics strongly influence disease risk[67]. *Which human diseases are most analogous to CE in dogs?* The classic answer has been IBD, which is, like CE in dogs, a category encompassing various chronic inflammatory conditions of the human gut, most notably CD and UC[68]. However, both human CD and UC

diverge from canine CE in almost all aspects (Table 1.1)[40, 69]. Specific histopathological criteria are required for diagnosis of CD and UC, distinguishing them from other disorders like microscopic colitis and Celiac disease. Treatment is divided into induction and maintenance phases, with clear, objective treatment endpoints including complete intestinal mucosal healing. Irritable bowel syndrome (IBS), a functional GI condition now referred to as a disorder of gut-brain interaction, may be more analogous to CE in dogs[70]. Irritable bowel syndrome affects between 10 and 25% of the global adult human population with a female predominance[71]. Clinical criteria include 3 days within the preceding 3 months of continuous abdominal pain or discomfort with at least 2 of the following: altered stool frequency or form, relief of discomfort

Table 1.1. Defining features of chronic canine (blue) and human (orange) intestinal conditions.

disease	**chronic enteropathy**	**Crohn's disease (CD)**	**ulcerative colitis (UC)**	**irritable bowel syndrome (IBS)**
diagnostic criteria	clinical criteria + exclusion of other causes	intestinal biopsy histology	intestinal biopsy histology	clinical criteria + exclusion of other causes
extra-GI manifestations		arthritis, dermatitis, osteoporosis, primary sclerosing cholangitis, episcleritis, delayed growth, anemia	see CD	anxiety, reduced quality of life
treatments	diet, antibiotics, immune suppression	immunomodulators, 5-ASAs, TNF antagonists; biologic agents	oral & topical immunomodulators, biologic agents	diet, lifestyle modifications, anxiolytics, motility modifiers, bile acid binders
complications		stricture, abscess, fistulae, perforation	colorectal cancer, fistulae	
references	Dandrieux 2019	Lichtenstein 2018	Dassopoulos 2015	Lacy 2021

with defecation, and onset of symptoms >6 months before diagnosis[72]. Symptoms determine IBS subtype: patients with diarrhea have IBS-D, those with constipation are IBS-C, and others fit into a mixed category. Like canine CE, the primary management strategy for IBS is dietary. The low fermentable oligosaccharides disaccharides monosaccharides and polyols (FODMAPs) diet, which limits consumption of poorly digested and absorbed carbohydrates, has been associated

with the best clinical outcomes[73]. This condition negatively impacts quality of life in affected individuals but is unlikely to result in mortality or impact longevity[74].

The distinction between IBD and IBS in people is clear. Histopathologic and endoscopic criteria, as well as non-invasive biomarkers such as C-reactive protein (CRP) and fecal calprotectin, distinguish individual patients likely to have IBD from those with IBS-driven signs[70,75]. In contrast, accurate means of determining the likelihood of inflammatory versus functional cause for GI signs in dogs have not been established. Serum CRP levels appear to be more relevant as a biomarker for PLE dogs[54,76], as most studies have failed to show a correlation between disease severity and CRP concentration in dogs with CE[17,77]. Fecal calprotectin concentrations may be higher in dogs with CE (study included PLE dogs) compared to healthy dogs[78], but data is lacking to prove the utility of this biomarker in establishing prognosis or guiding therapy[79]. At this point, it is unclear if CE in dogs is a more appropriate model for IBS or IBD seen in human patients. The growing prevalence of each of these conditions[80,81,82] serves as a motivating factor to advance understanding of canine CE so well-powered studies with translational potential can be designed and completed.

1.2 Pathophysiology of Canine Chronic Enteropathy – Current Understanding

The pathophysiologic basis of CE in dogs is not well understood. Current theories center around disruption of normal interactions between the host intestinal immune system, the intestinal microbiota, and environmental factors[1,2,83]. However, most explanations are speculative extrapolations from findings in studies of mice and humans. The remainder of this section will review the existing data regarding canine CE pathogenesis and highlight key knowledge gaps.

Genetic Background of CE

The elevated prevalence of CE in certain breeds suggests a genetic component to disease[10]. A study of dogs in the United Kingdom found that Weimaraners, Rottweilers, border collies, and boxers were at increased risk of CE development[8]. German shepherd dogs (GSD) are also overrepresented among CE dogs[9] and have been the most extensively studied. This breed appears to be somewhat of an immunological outlier, as evidenced by their predisposition for various infections (e.g., sinonasal and systemic aspergillosis, bacterial endocarditis) and autoimmune conditions (e.g., perianal fistulae, lupus erythematosus). In line with this, GSDs are overrepresented within the antibiotic-responsive CE subgroup German 2003[1,8,23,84]. The precise driver of these dogs' challenges with microbes that rarely cause disease in non-GSD and are ubiquitous in the environment is unclear, though genome wide association studies have identified risk association for CE with Toll-like receptor 5 (TLR5) single nucleotide polymorphisms (SNPs)[85]. Additionally, analysis of SNPs in nucleotide oligomerization domain 2 (NOD2), one of the most implicated genes in human CD, pointed to the heterozygote genotype for all four SNPs being significantly more common in GSDs with CE than in control GSDs[86]. This distinction did not hold true in non-GSDs with CE. Another study found a higher expression of TLR4 and reduced expression of TLR5 in the duodenum, ileum, and colon of CE-affected GSDs compared to healthy Greyhounds[87]. This pattern of differential expression was not seen in a study comparing various breeds of dogs with CE to healthy beagles[88]. Thus, genetic contributions may set GSDs up for a unique subtype of CE. Most likely, CE (or the multiple unique conditions encompassed by this diagnosis) is polygenic, with diverse genetic risk factors contributing to disease risk in different breeds.

Immunological Characterization of CE

The intestinal tract is considered the largest immune organ as it is home to the body's largest number of lymphocytes and macrophages; it also is the greatest producer of

immunoglobulins. The gut-associated lymphoid tissues (GALT) make up a huge proportion of the mucosa-associated lymphoid tissues, which are secondary lymphoid centers interspersed within barrier tissues. One unique aspect of GALT is their lack of afferent lymphatics. They directly sample antigens from the lumen via a specialized follicle-associated epithelium that covers them. A diverse mix of immune cells congregate below this subepithelial dome, allowing for antigen presentation and subsequent cellular reactions. Throughout the vast surface area of the intestinal mucosa that is *not* occupied by GALT, intestinal epithelial cells (IECs) are knit together to create a tight barrier separating the outside world from host tissue. Between and directly beneath the single cell layer of IECs are immune, stromal, lymphatic, and endothelial cells which support, protect, and nourish the gut barrier. All these cell types work together to accomplish the challenging jobs of the GI tract: digesting and absorbing nutrients, maintaining commensal microbial populations, avoiding overzealous reactions to dietary and microbial antigens, and mounting rapid, effective immune responses against pathogens. Given the many players and the dynamic nature of this organ system, it is not surprising that characterizing it in health and disease is a fraught endeavor.

Much work has been dedicated to histopathological evaluation and classification of the most relevant tissues in CE – intestinal mucosal biopsy samples. This necessarily involves establishment of the 'normal' histopathological appearance of GI mucosa. Typically, this involves collection of samples from healthy dogs without obvious indicators of GI disease, such as inability to gain weight, diarrhea, or frequent vomiting. Beyond this, it becomes challenging, as there are no tests to ensure GI tract "normalcy". Unlike renal disease, for example, where a creatinine above a certain cut off correlates with a reduced glomerular filtration capacity, there are no analogous markers for the gut. Moreover, since veterinary patients cannot verbally communicate to their caretakers the presence of more subtle clinical signs like abdominal pain or nausea, the burden of GI dysfunction may be underestimated. Moreover, there are known differences between breeds of dogs. Larger dogs have also been shown to have longer GI

transit time, higher fecal moisture, and increased dry matter digestibility compared to smaller breed dogs[89]. A dog's height at the shoulder correlates positively with reduced stool form[90]. Other breed-associated aspects of GI habits are not based in any data, such as the idea that it is normal for toy or Nordic breeds to be picky eaters. Thus, objective criteria stipulating ensuring normal GI tract function across the diverse spectrum of pet dogs are non-existent. Considering this, most studies utilizing a control population have included young, apparently healthy colony dogs of a single breed (most often beagle or hound dog) eating a common commercial diet[91,92,93]. The homogeneity of these groups, compared to more varied client-owned CE affected dogs, could skew comparisons. Older studies found that adult purpose-bred beagles had increased bacteria in duodenal juice compared to healthy adult non-beagles, with bacterial levels correlating with an indirect marker of intestinal permeability[94]. Breed is also known to influence gut bacterial populations among dogs with CE, further suggesting that breed-matched controls may be important[87]. Another source of healthy controls has been dogs of various ages and breeds being euthanized for a non-GI reason[95,96,97]. A thorough a medical history to rule out previous or current CE signs, as well as medications impacting the GI tract, are not always available in these dogs. For comparison, control populations for human studies involving histologic evaluation of intestinal tissues typically consist of healthy adults undergoing endoscopy for colorectal cancer screening or for investigation of gastroesophageal reflux[98,99,100]. Revisiting definitions of health and normalcy in the canine GI tract is thus an important consideration.

There is no precise histologic definition of CE in dogs. The inflammatory cellular infiltrate in CE may vary, both in terms of cell types and anatomic location (epithelial vs. LP). Most commonly, lymphoplasmacytic inflammation dominates in the intraepithelial and LP compartments. Fewer numbers of neutrophils, eosinophils, and macrophages are often present. In line with non-uniform clinical presentations and responses to therapy, varied histopathological findings provide another reflection of the heterogeneous mix comprising CE patients.

Histopathology is a necessarily subjective tool, relying heavily on human perceptions. To reduce some of this subjectivity, the first guidelines for assessment of GI tissues were established in 2008 by the World Small Animal Veterinary Association (WSAVA) Gastrointestinal Standardization Group[101]. Their goal was to encourage standardized assessment of GI biopsies to advance the study of GI diseases and enable comparison of histopathologic findings between studies. Pathologists were tasked with describing the severity and cellular makeup of inflammatory infiltrates, as well as identifying and grading morphological lesions. Updated WSAVA guidelines incorporated grading the severity of intraepithelial lymphocyte infiltration as well as lamina propria (LP) lymphocyte, neutrophil, and eosinophil presence[22].

When comparing duodenal mucosal samples from healthy dogs to those with CE, keeping in mind the caveats just discussed, two main categories of histological differences have been compared: inflammatory and morphological lesions[101]. Total inflammatory cell density is comparable in the duodenal LP in CE vs. health, but cell density may be greater in the intraepithelial compartment of CE dogs[84]. Increases in IgG-producing plasma cells and higher expression of MHC class II was documented in the duodenal LP in dogs with CE compared to healthy[84]. Morphological or architectural changes, including epithelial injury, crypt lesions, villous atrophy, fibrosis, and lacteal dilatation, may also distinguish healthy from CE tissues. Older guidelines published by the WSAVA recommended assessment of villous stunting, epithelial injury, crypt distension, lacteal dilation, and mucosal fibrosis[22]. An updated, simplified histopathologic scoring system removed lacteal dilatation and mucosal fibrosis as interobserver variation was excessively high[102,103]. Later, Allenspach and colleagues developed an even further simplified scoring system which excluded assessment of mucosal fibrosis due to lack of consensus among pathologists[104].

Despite many attempts to improve GI biopsy grading schemes, the utility of this invasive diagnostic test remains debatable. Various studies have attempted but failed to link disease activity to histopathologic lesion severity[17,78,88,105,106]. Even with the updated histopathologic

scoring system developed by Allenspach et. al., correlation between CIBDAI/CCECAI and summative duodenal histologic score was barely moderate (r = 0.42). When considering individual components, the strongest significant correlation was for LP neutrophils and was nonetheless moderate (r = 0.45). While many studies simply state that their healthy control populations all had "histologically normal" biopsy samples[91], others indicate mild to moderate abnormalities within control patients[107,108,109]. This casts doubt as to the role of histopathologic evaluation in "diagnosing" CE, as biopsies may be identical in health or disease. Longitudinal studies have failed to associate resolution of histologic "lesions" with positive responses to therapeutic interventions[24,105,110]. This could be due to lack of association of evaluated histological features with clinical disease (i.e., aspects deemed abnormal at the time of diagnosis may be normal for that dog). Alternatively, changes may occur in the intestinal mucosa that are not evident to the pathologist. One study utilizing transmission electron microscopy detected improvements in brush border lesions with dietary therapy that correlated with clinical improvements[52]. Thus, more sensitive techniques may be necessary to appreciate intestinal healing. It is also possible that histological changes lag behind clinical improvement. In dogs with *E. coli*-associated granulomatous colitis, PAS-positive macrophage infiltrates take much longer to resolve than large bowel diarrhea and colonoscopic lesions[111]. Overall, the lack of associations between disease severity and prognosis and histopathological features questions the relevance of GI biopsy histology. Other approaches are needed to advance understanding of CE in dogs.

Challenges of histopathologic evaluation of GI tissues aside, the GI immune system is nonetheless implicated in the pathogenesis of canine CE. Overactivity of the innate immune system has been hypothesized, and one study found upregulation of TLR2 expression in the duodenal mucosa of 20 dogs with CIE compared to 7 healthy beagles[88]. Expression of TLR2 also correlated with clinical disease severity (CCECAI). Other studies have documented greater mRNA expression of duodenal and colonic TLR2, TLR4 and TLR9 in dogs with CE responding

to steroids compared to healthy beagle dogs[112]; no changes were seen with achievement of clinical remission. Neutrophils appear to play a role in CE. The circulating neutrophil-to-lymphocyte ratio is associated with disease severity in one study, however, half of the dogs included had PLE[113]. Duodenal LP neutrophils correlated moderately with CCECAI in a study of 42 CE dogs (7 of which hypoalbuminemic)[104]. The correlation between concentrations of fecal calprotectin, which accounts for 60% of the cytosolic protein within neutrophils[114], and disease severity in a cohort of dogs with CE (50% of which hypoalbuminemic) also supports a role for intestinal infiltrative neutrophils in these dogs[79]. Other investigations have documented higher activated NFκB mRNA expression in duodenal tissues from CE dogs compared to healthy beagles. After 4 (diet) or 10 (immunosuppressant) weeks of treatment, histologic scores were unchanged, but NFκB expression decreased in line with clinical improvements[115]. Most if not all the studies involving the role of the innate immune system in canine CE are limited in scope, involving small patient populations and one to a handful of cytokines or cell types. These limitations preclude generation of far-reaching conclusions regarding the role of the innate immune dysregulation in CE in dogs.

Contributions from adaptive immunity to CE pathogenesis have also been investigated. German Shepherds again have been the most studied breed, with initial data pointing to a relative deficiency in mucosal IgA production[116]. In CE dogs of various breeds in Japan (62% of which hypoalbuminemic) not responding to diet or antibiotics, reduced IgA within duodenal mucosal and fecal samples was identified relative to a group of 20 healthy Beagle dogs[117]. Chronic enteropathy dogs in this study also had lower duodenal TGFβ expression[117]. Interestingly, older studies found *higher* numbers of IgA-producing plasma cells in endoscopic biopsies from dogs with chronic non-specific gastroenteritis compared to healthy control dogs or dogs with histologic lesions of CE[91]; this same study also identified fewer villus T cells in CIE dogs compared to healthy. In line with this concept of concurrent pro- and anti-inflammatory

signals in the gut mucosa, Garden et. al. identified colocalization of IL-10 and IFNγ in cells infiltrating the intestinal mucosa of dogs with CE[94]. Investigations of duodenal $CD4^+$ helper T cell populations have been unsuccessful in ascribing CE pathogenesis to a canonical T helper cell subset[84]. Some describe CE as being characterized by a "mixed" Th1/Th2 response[4]. However, this is likely a moot point as these previously heralded cell profiles are now seen as oversimplifications which are not reflected *in vivo*[118].

Numerous studies of duodenal mucosal cytokine expression have been completed, without a unifying consensus as to a characteristic CE cytokine signature. Findings that have been replicated by more than one study include an increased expression of IL-12 and reduced expression of IL-10 in the SI mucosa of CE dogs compared to healthy[119,120,121]. One recent study found increased duodenal expression of IL-1β in CIE dogs with CCECAI scores ≥9 compared to healthy dogs and dogs with PLE[33]. Based on the evidence to date, it is difficult to conclude that upregulation of pro-inflammatory cytokines is present in the intestinal mucosa of CE dogs. These findings coincide with highly variable results from histopathologic comparisons of T cell and plasma cells numbers in intestinal mucosal samples from dogs with CE vs. healthy dogs[84,91,122,123,124].

The past 2 decades have seen the application of more advanced immunological techniques to the study of CE in dogs. Flow cytometry has improved resolution of inflammatory cells within intestinal biopsies beyond what is evident with light microscopy. An initial study of 10 healthy young adult mixed breed dogs found that $CD45^+$ cells comprised 60% among cells digested from SI mucosal biopsies, with 50 to 66% αβ (alpha-beta) T cells and 30 to 38% γδ (gamma-delta) T cells[107]. Several different groups have found that most intraepithelial lymphocytes (IELs) are $CD8^+$[107,108,125,126]. In contrast to German et. al.'s 2001 immunofluorescence-based study, using flow cytometry, Haas and colleagues found greater numbers of γδ IELs in endoscopic biopsies from CE dogs compared to samples from client-

owned healthy dogs[108]. One study of peripheral blood leukocyte populations found lower proportions of γδ T cells in 19 CE compared to 6 healthy dogs[127]. No differences in prevalence between health and disease were seen in IELs expressing the CD8αα homodimer, a feature of non-conventional, regulatory IELs[128]. Immunohistochemical investigation of $Foxp3^+$ cells, assumed to be regulatory T cells (T_{reg}), identified reduced numbers in CE duodenal villi compared to tissues from healthy control dogs of various breeds; this led investigators to postulate that a primary defect in T_{reg} homeostasis could promote or support chronic intestinal inflammation in dogs with CE[109,121], a theory that needs to be further explored. All in all, these types of studies underscore the importance and challenges of accurate phenotyping of intestinal T cells, which hinges upon accurate discriminatory markers, as well as canine-specific reagents. Studies in other species have exhibited a huge diversity of T cells (all of which may be CD3+) within the intestinal mucosa[129,130,118], underscoring the need for high-resolution methods of cell subtype evaluation.

Relatively few studies have investigated the immunological contributions of intestinal epithelial cells, despite long-standing evidence that they are more than a monomorphic population of barrier cells[131,132]. Intestinal epithelial cell expression of IL-10 and IFNγ was documented in Irish setters and beagles using mRNA in situ hybridization[94]. More recently, the cytokine repertoire of canine IECs has been expanded to include IL-25, IL-33, and thymic stromal lymphopoietin (TLSP)[133]. Other studies in rats and humans have demonstrated the antigen presenting capabilities of enterocytes[134,135]. Studies of canine tissues are limited in this regard to ones documenting epithelial expression of MHC class II[95], which is increased in enterocytes of dogs with *E. coli*-associated GC but unknown in CE dogs[136]. Higher NFκB expression by intestinal epithelial cells of dogs responding to diet (with positivity decreasing after diet change) compared to those responding to immune suppression has been

documented[115]. Additional work is needed to explore IEC contributions to canine CE pathogenesis.

Additions to Understanding of CE from Studies of the Intestinal Microbiota

Study of cells and cytokines are important but represent a single component of the physiology underlying GI homeostasis. The pathogenesis of chronic inflammatory intestinal disorders cannot be extricated from intestinal lumen inhabitants. The GI microbiota is defined as the community of microorganisms inhabiting the GI tract, including bacteria, viruses, fungi, archeae, and others[137]. This is by no means a new area of study[138] but has received a great deal of attention in recent years, facilitated by a marked decline in the price of next generation sequencing based analyses. Gut microbes have far-reaching roles from vitamin synthesis to education of the immune system to energy metabolism[139]. Specifically, the GI microbiota has been linked to maintenance of immune tolerance, a robust GI barrier, and nutrient digestion and absorption[140].

One explanation of CE in dogs involves bacterial community shifts or individual enteropathogens triggering injurious GI mucosal immune responses. Thus, many groups have evaluated the bacterial taxonomic makeup in fecal samples and on intestinal mucosal surfaces of dogs with CE. These studies attempt to define microbial constituents characteristic of health and of disease. Review of these studies highlights a few recurrent themes. The fecal microbiota of CE dogs may exhibit reduced α-diversity and distinct β-diversity compared to healthy[141,142,143,144]. Increased relative abundance of *Proteobacteria* and reduced relative abundance of *Faecalibacterium* have also been reported[142,147,148]. However, results from other studies involving well characterized patients have failed to identify significant differences in the composition of fecal microbial communities between healthy and CE dogs[42,145,146]. It is possible that fecal microbial populations are not most representative of the most disease relevant gut cohabitants. The mucosal microbiota is more relevant to the intestinal immune system, given spatial proximity. Duodenal mucosal bacterial populations in CE have been compared to those

from healthy dog samples in a handful of studies. Conflicting evidence exist regarding species richness in duodenal mucosal bacterial populations, with some finding lower richness in CE[149] and others finding no differences[144,150]. Increased relative abundances of mucosal *Enterobacteriaceae* was appreciated in studies utilizing fluorescence in situ hybridization[151] and 454-pyrosequencing[150]. Within dogs with CE, no distinct duodenal mucosa bacterial signatures have been identified in CE dogs responding to diet vs. immunosuppressants[152]. Thus, despite concerted efforts, no unifying CE-associated microbiota has been described.

Much discussion of the microbiota in CE revolves around dysbiosis. This is defined as any deviation from the "normal" GI microbiota taxonomic composition, either in diversity or differentially abundant taxa or their combination. Dysbiosis has a negative connotation[153,154], but this may be an oversimplification. A fecal microbiota "dysbiosis index" (DI) to assess dogs with CE has been established[155]; this assigns a single numerical score to bacteria present in a fecal sample, with values less than 0 being considered normal and values >2 classified as dysbiotic. This has been included as part of the analysis in numerous studies of dogs with CE[42,156,157,158,159]. In some studies, statistically significant differences are seen between healthy and CE fecal samples, but in others, no differences are evident. The fecal DI may or may not normalize with achievement of clinical remission[42,158,160] and is influenced by diet[161],[162]. Thus, the DI fails to guide treatment or prognosis for dogs with CE. It may be used to screen dogs for use as fecal microbiota transplant (FMT) donors, if an FMT from a donor with a negative (normal) DI value would prove more beneficial. In humans, FMT donor material (including ability to engraft and presence or absence of select bacteria) can influence treatment success in human patients treated with FMT[163,164]. However, this has not been studied rigorously in dogs. Various other commercial entities offer fecal microbiota analysis, and the clinical utility of their readouts is dubious, as many microbial communities may be compatible with health or disease in a specific individual.

Regardless, accurately "defining" the microbiota characteristic of dogs with CE may be a futile endeavor as studies of the human GI microbiota have questioned the concept of a signature microbiota that precipitates or is tightly linked to disease[165]. The ability for certain microbes or groups of microbes to trigger disease largely depends on the individual host, and therefore various communities of microorganisms could be compatible with health or disease[166]. In this way, a focus on microbial functions rather than identities could be more informative; studies evaluating the canine fecal metagenome are limited but have indicated that fecal bacterial transcriptional activities are influenced by dietary fiber content[60,167].

An alternative hypothesis regarding CE pathogenesis posits a loss of oral tolerance toward "normal" GI bacteria triggering disease. The heightened immunoglobulin coating of fecal bacteria from CE dogs compared to healthy dogs, alongside largely similar fecal microbial community makeup between these populations, lends weight to this theory[145,168]. It is also possible that altered gut barrier function disrupts normal conditions in the intestinal lumen (e.g., increases oxygen concentrations), and results in depletion of obligate anaerobes and expansion of unfamiliar microbes[169]. Objective quantification of intestinal permeability is technically challenging[170], and data proving CE dogs have heightened intestinal permeability is lacking[171,172]. At this point, there are many theories linking gut bacterial inhabitants to intestinal disease, however, we lack data necessary to draw conclusions regarding the GI microbiota's role in predicting, initiating, or preventing CE in dogs.

Possible Roles for Metabolites in Canine CE

Metabolomic and proteomic studies offer a step closer to determining what is happening in the intestinal lumen and how that might impact the host. Untargeted approaches enable identification of a wide array of metabolites. Investigation of serum metabolites in CE versus health is limited; one study found higher gluconic acid lactone, hexuronicacid (ascorbic acid), 3-hydroxybuta-noic acid and ribose in diseased dogs[142]. Serum or plasma amino acids appear to

more dysregulated in PLE compared to normalbuminemic CE dogs, but one study of 10 CE dogs (6 of which had serum albumin >2.6 g/dL) found lower concentrations of serine in plasma samples, which correlated negatively with CCECAI[77]. The largest study to date comparing sera from 55 CE to 204 healthy dogs also identified lower total circulating fatty acids, as well as lower glycine, and higher phenylalanine and lactate levels using a canine-specific nuclear magnetic resonance spectroscopy platform[173]. A single study of the serum proteome of CE dogs (n=9) compared to healthy control dogs (n=16) identified 45 differentially abundant proteins; results pointed to disrupted lipid metabolism and enrichment of acute phase proteins in CE[174].

More investigation has focused on fecal metabolomics. An early fecal untargeted metabolomics study comparing fecal samples from 15 dogs with “GI inflammation” to 15 healthy dogs published in abstract form only highlighted elevations in primary bile acids (BAs) and reduced levels of secondary fecal BAs[175]. Subsequently, various studies have shown reduced concentrations of secondary BAs and increased concentrations of primary BAs in feces from CE dogs compared to healthy[156,159,176]. Fecal secondary BA concentrations were also found to rise in CE dogs responding to a hydrolyzed protein diet; these changes were not seen in the non-diet responsive group[42]. A small study utilizing TMT-based fecal proteomics identified differential recycling of BAs based on reactome analysis in the food-responsive CE dogs[177].

Other metabolites have been implicated in CE. Minamoto and colleagues found lower total fecal short chain fatty acid (SCFA) concentrations, specifically acetate and propionate, in the feces of diseased dogs compared client-owned healthy dogs[157]. Another small study utilizing untargeted metabolomics found that 9 dogs with steroid-responsive CE had elevated fecal levels of various amino acids, including tryptophan, and glucose compared to 13 healthy dogs[158]. As with other investigations of CE, many of the proteomic and metabolomic studies carried out to date mixed in PLE dogs with the CE population, which muddles the significance of identified compounds. Still, these are two promising techniques whose results may inspire new hypotheses regarding CE pathogenesis, monitoring, and treatment.

Much effort has been exerted toward defining the dysfunction underlying canine CE. This has provided a foundation of knowledge on which to base future studies. Under current criteria, CE encompasses a wide range of patients for which methods of subcategorization are lacking. Until practical disease subcategories are defined, it will likely be impossible to achieve an accurate explanation for what distinguishes healthy dogs from those with CE. Luckily, most dogs do not behave like humans with CD or UC and can have clinical remission with tolerable management strategies. Dietary intervention warrants further exploration as this is the most effective treatment strategy thus far.

1.3 Special Focus on the Role of Diet in Management of CE

Overview of Diet's Role in Intestinal Homeostasis

The study of both canine and human chronic intestinal conditions has largely focused on host-related factors: genetic background, serum biomarker levels, histopathological lesions, and so on. However, environmental factors are of critical importance for the G tract, an organ that is a conduit for the outside world coursing through the body from mouth to anus. Alongside drug exposures, pollution, and infectious agent exposure, diet is one of the most powerful environmental factors influencing intestinal health and disease. The complexity of the GI tract is underscored by its concomitant jobs, many of which are intimately linked to nutrient acquisition. The gut must simultaneously move and break down life-sustaining nutrient sources, assimilate luminal nutrients, screen for potential pathogens (food-borne and otherwise), and extract enough water to keep the host hydrated. Both the host as a whole and the enterocytes themselves gain nutrition from luminal foods.

Besides providing the host with the energy to sustain life, diet is also remarkably influential on the host immune system. Material derived from foods may have the direct and indirect impacts on the GI immune system. The most common response to diet-derived soluble antigens is one of oral tolerance[178], meaning the host does not mount an immunological

response against the non-self antigens despite lack of central (thymic) tolerance[179]. This appears to be orchestrated by dendritic cell (DC) sampling of intestinal lumen contents. Intestinal DCs are distinct from DCs elsewhere in the body given their tolerogenic nature. This is fostered by IEC-produced TSLP, TGF-β and retinoic acid (RA)[180]. Dendritic cell sampling is highly efficient; food-derived antigen may be detected in the intestinal epithelium within 1 minute of feeding[181]. Induction of oral tolerance furthermore involves cooperation between mucosal and mesenteric lymph node (mLN) cell populations; intestinal DCs shuttle antigen to mLN in response to CCR7 signaling[182], where they promote generation of T_{regs}[183]. Homing back to the intestinal LP for expansion and maintenance is key for fulfillment of oral tolerance[178]. Evidence from mouse studies indicate that the primary driver of immunological development in the SI is food-derived antigen, while colonic GALT is most strongly influenced by microbiota-derived antigens[184,185]. A unique aspect of intestinal mucosal-mediated tolerance is the ability of that tolerance to extend beyond the gut into the periphery. Oral ovalbumin administration to mice prevents systemic immune reaction to subcutaneous injection 2 weeks later[186]. How this occurs is not fully understood but it appears to require T_{reg} participation[187]. The exact details of oral tolerance generation are incompletely delineated in dogs, but they are assumed to occur via similar mechanisms as in other species.

A breakdown of oral tolerance has been implicated in the pathogenesis of various diseases including Celiac disease[188]. One mechanism for loss of oral tolerance to food antigens is reduced digestive capacity resulting in increased antigenicity of food items[189]. Normal gastric and proximal small intestinal digestive processes result in proteins being of polypeptide size within most of the SI lumen. Polypeptides should have fewer epitopes than native, undigested protein, but may still be immunogenic[190]. Additionally, some proteins (i.e., those within legumes, dairy products) are resistant to mammalian digestive enzymes. Digestive capacity may also be reduced in dogs with CE. Reduced SI surface area due to villous damage, reduced expression of brush border digestive enzymes and carriers have been implicated could all result in

maldigestion[191]. Medications, specifically those suppressing gastric acid production, may also thwart normal digestive capacities as acidic pH is needed for optimal pepsin activity[192]. Thus, intrinsic and extrinsic factors may alter the immunological potential of diet components. Altered skin barrier function has been conclusively linked to increased incidence of food allergies in people[193] and is highly suspected in dogs[194]. The role of intestinal permeability on development of food allergies is less well established in humans and unknown in dogs[195]. It should be noted that none of these mechanisms have been conclusively proven in dogs, let alone dogs with CE.

Classical food allergy involving IgE-mediated hypersensitivity is thought to affect a minority of dogs with CE, given the anamnesis of clinical signs, lack of Th2 signature, and ability for many CE dogs to return to their previous diet without recrudescence of disease[17,196]. In line with this, available serum tests quantifying IgE titers against various food components do not correlate with foods that provoke adverse clinical signs (typically GI or dermatologic) in dogs[197,198]. Non-IgE mediated adverse food reactions (AFR) or food intolerances are probably more prevalent among dogs with CE; these may or may not involve aberrant immune reactions[199]. Non-IgE mediated food allergies in humans are thought to involve allergen-specific T cells, eosinophils, and other players[200]. Kathrani et. al. documented reduced TNFα and IL-10 production when whole blood from dogs with CE was incubated with hydrolyzed protein diets compared to commercial intact protein diets, suggesting reduced immunogenicity of the hydrolysate[201]. Culture of peripheral blood mononuclear cells with hydrolyzed diet extracts documented activation of T cells *in vitro* in about 25% of samples from 316 dogs[202]. While neither of these experimental models come close to recapitulating the gut mucosal environment, they suggest that proteins within hydrolyzed diets may still be immunogenic. In line with a possible role for eosinophils in dogs with CE, one study found elevated serum levels of 3-bromotyrosine (a marker of eosinophil activation) in CE dogs (both steroid and diet responders) compared to healthy dogs[203]. Although it seems plausible that foods provoke aberrant immune reactions in dogs with CE, much more work is needed to prove or disprove this.

Understanding of non-immune mediated AFR in dogs is quite limited[54, 204]. Foods may drive clinical signs through toxicity (contamination or spoilage) or pharmacological reactions (histamine content). Phenomenon such as carbohydrate intolerance could be relevant for dogs with CE. Lactose intolerance is a classic example of this; it is rare in dogs, likely due to the small amounts of milk products typically consumed by adult dogs[205]. Carbohydrates may invoke clinical signs in other ways. Taking the example of the low FODMAPs diet for human IBS patients, a nutritional strategy which excludes various types of carbohydrates that helps significantly reduce clinical signs in 52 to 86% of patients[206]. For a time, the primary postulated mechanism of action was through exclusion of carbohydrates that are poorly absorbed in the SI, preventing excessive fermentation with subsequent osmotic effects and gas production by colonic microbes[207]. Current data suggests that the digestive outcomes of FODMAPs carbohydrates are comparable in healthy and IBS individuals, but that IBS individuals experience increased visceral pain from the excess liquid and gas in the colonic lumen[208]. More recent studies have documented reduced levels of circulating pro-inflammatory cytokines in IBS patients on a low FODMAPs diet[209], but further study is needed regarding the immunomodulatory properties of this diet.

The potential contribution of FODMAPs to CE in dogs is unknown. Common carbohydrate sources in commercial dog foods include brewers rice, oats, and corn, all of which are low FODMAPs. Other common ingredients like barley and wheat are high in FODMAPs and therefore could be implicated in CE clinical signs. Presumably, the grain-free fad would have reduced the FODMAP content of dog foods, however, many recipes replaced typical carbohydrate sources with legumes, which are high in FODMAPs. Therefore, grain-free commercial canine diets may exacerbate clinical signs dogs with carbohydrate intolerances if they exist. Diet components other than proteins may therefore influence disease manifestations, and additional work is needed to unravel the immunological and non-immunological mechanisms underlying adverse food reactions.

Another dietary strategy associated with powerful disease modulating effects is exclusive enteral nutrition (EEN). This strategy involves provision of caloric needs to the patient via a liquid formula sourcing nutrients from simple building blocks[210]. Protein is provided in polymeric (intact whole protein) or elemental (individual amino acids) form, and carbohydrate and fat are provided in readily assimilable means such as starches (which may be hydrolyzed) and medium chain triglycerides, respectively[211]. Typically, the diets are devoid of or very low in fiber. Both elemental and polymeric diets are associated with achievement of clinical remission and mucosal healing in 60 to 80% of pediatric CD patients[212,213]. The exact mechanisms of action of this type of nutrition are unknown, but appear to involve shifts in GI microbiota taxonomy, correction of nutritional deficiencies, and anti-inflammatory effects through antigen exclusion and restitution of gut barrier functions[214]. A major impediment to more widespread use of EEN is patient compliance. Pediatric patients are often managed with a nasoesophageal feeding tube for the duration of therapy[210]. Canine hydrolyzed protein diets have attempted to achieve some of the aims of EEN through chemical and heat destruction of proteins, resulting in molecules that should be easily digested and absorbed, and too small to cross-link IgE[215]. However, hydrolyzed diets do not benefit all dogs with CE, which may be due to persistent antigenicity, intolerable fat content, or other factors[216,202]. Extensive processing of foods (inherent to essentially all cooked commercial dog foods) may also reveal previously hidden antigenic epitopes and elevate advanced glycation end-product concentrations, both of which could exacerbate GI disease[217,218]. Until recently, a diet akin to formulae utilized in EEN formulated specifically for dogs has not been available.

Diet macronutrient composition may also influence intestinal homeostasis. Fats are complicated to digest, requiring coordination of digestive enzymes, bile acids, micelle formation, and the lymphatic system[219]. Fat maldigestion and malabsorption can result in several clinical outcomes including colonic fluid secretion, bloating, and dysregulation of mucosal immunity[199]. Dietary fat slows gastric emptying, and so fat content also has a major role in GI motility

patterns[44]. Dogs and humans with intestinal lymphangiectasia (IL) may experience marked reduction of disease activity with reduced dietary fat content[36,220]; intestinal lymphangiectasia may contribute to CE but is more well recognized as a leading cause of PLE[32]. Still, many dogs with CE seem to do better clinically on diets with lower fat contents.

Carbohydrates that are not digestible by mammalian enzymes make up dietary fiber. Fiber modulation has been used for decades in the management of bowel conditions due to its far-reaching effects on gut motility, digestibility, and ability to serve as a prebiotic[221]. The macronutrients of protein, fat, and carbohydrate are interrelated parts of the diet, and shifting one component may in turn result in differential impacts on another component. Thus, there are many moving parts in dietary modification that work together to account for the individual patient's response.

Taken together, distinct but interrelated aspects of diet have the power to shape GI tract function and dysfunction. With more advanced tools, our ability to assess the impact of diet on intestinal physiology is greater than ever. Studies involving representative samples of CE dogs are necessary to see if the conclusions reached based on *in vitro* data, mouse models and investigations of human diseases apply to these patients.

Clinical Data Regarding Dietary Intervention for Dogs with CE

Of all the questions regarding in CE in dogs, the one that seems to have been most extensively explored and answered surrounds the efficacy of dietary therapy for achieving and maintaining clinical remission. Diet as monotherapy for dogs with CE has gained traction over time. Allenspach et. al.'s 2007 publication reporting achievement of clinical remission in 66% (39/65) of dogs with CE clinical signs achieved remission with diet change (to salmon based limited ingredient commercial kibble) was initially an outlier[17]. Subsequently, other studies documented similarly high response rates[11,42,48,106,222], ranging from 56 to 88%. *Are any factors predictive of a favorable response to dietary therapy for dogs with CE?* Earlier studies indicated

older dogs with clinical signs localized to the SI were *less* likely to respond to diet[17,24], but this association has been contradicted by findings of other studies[10,145]. Other groups have concluded that dogs responsive to diet typically have less severe disease (lower CIBDAI/CCECAI) at diagnosis[11,17,47]. However, this is not a consistent finding across studies either[24,48]. It should be pointed out the large potential for bias given the treatment-trial approach to CE. Older treatment algorithms suggested a multimodal (e.g., diet + antibiotic + immunosuppressant) approach in more severely affected dogs[2]. This could artificially reduce the proportion of dogs classified as diet-responsive as they never experienced diet as monotherapy.

What is the ideal diet for a dog with CE? The answer to that question is not apparent and likely never will be, due to several factors including the heterogeneity of the CE population. Clinical remission has been documented in dogs ingesting diets formulated to be highly digestible[106] or with different ingredients than their baseline diet[17,24], hydrolyzed protein diets[42, 49,52,106,223], diets enriched with fiber[43,224], and home-prepared diets[160]. Ultra low-fat (<1.5 gm fat per 100 kCal metabolizable energy) diets have the most evidence in management of dogs with PLE[220,225,226], but may also benefit normalbuminemic CE dogs. Thus, a wide array of diets may benefit an individual dog with CE. This brings up another limitation existing data on treatment efficacy in CE dogs: most studies exclude the possibility of diet responsive CE if the patient does not achieve clinical remission after exclusive feeding of a single diet for 2 to 3 weeks. Over the counter and veterinary therapeutic diets vary widely in terms of ingredients, digestibility, macronutrient profile, and format. While there are hints that might suggest a particular dietary strategy for a particular patient, trial-and-error dominates the approach. Thus, current recommendations involve instituting multiple diet trials before deeming a dog with CE non-diet responsive[44]. It is not uncommon to encounter patients previously deemed “unresponsive” to diet who ultimately achieve remission with diet. The spectrum of available, high quality, complete and balanced diets thus influence the proportion of dogs with CE ultimately classified as diet-responsive.

As CE tends to be a relapsing, episodic disease, equally important as initial response to diet is the long-term clinical response to this intervention. In a prospective clinical trial tracking 203 dogs with CE over 6 to 12 months post-diagnosis, diet was the *only* therapy associated with stable remission[23]. As seen by other groups, CE dogs that initially respond to antibiotics or immune suppressants typically have an unacceptable number of relapses and breakthrough clinical signs[10,11,17,40]. One study found that 79% of diet responsive CE dogs were able return to eating their original diet without relapse[17]. This points to a more lasting positive impact from diet intervention and argues against presence of true food allergy. In practice, dogs are often maintained on the diet that brought about remission due to fear of clinical deterioration, particularly for more severely affected dogs. On the contrary, dogs with antibiotic-responsive CE almost invariably have recurrence of diarrhea with antibiotic cessation[45,227]. Dogs with immunosuppressant-responsive CE often have relapses despite medications and/or unacceptable side effects that require tapering or discontinuation of the drug(s). Diet is therefore the most successful treatment intervention identified to date for CE dogs.

Putative Mechanisms for Diet-Responsive CE

Why and how various diets help a large proportion of dogs with CE achieve clinical remission is unclear. One theory is that intestinal mucosal inflammation decreases due to reduced antigenic stimulation[228]. However, this has not been decisively proven, despite numerous efforts evaluating intestinal biopsy histopathology before and after dietary intervention[25,52,105,229]. The proportion of CE dogs positive for one putative marker of autoimmunity, perinuclear anti-neutrophil cytoplasmic antibody (pANCA), increased from 62 to 81% despite clinical improvement in 39 diet responsive CE dogs[24]. Changes in specific inflammatory cell subtypes may be influenced by diet intervention. For example, Dandrieux et. al. documented an increase in the ratio of calprotectin positive to $CD163^{+}$ macrophages within the duodenal mucosa concurrently with positive response to diet in 10 CE dogs[230]; this brought

the ratio closer to that seen in healthy control tissues. The only longitudinal study evaluating gene expression in duodenal biopsy samples with RT-PCR was underpowered (n=12 dogs with CE before and after diet +/- probiotic); no difference in expression of various toll-like receptors, pro-inflammatory cytokines or trefoil factors was noted[229]. Otherwise, longitudinal studies are lacking, leaving many unanswered questions regarding the impact of diet in these dogs.

Diet associated restoration of intestinal barrier structure and function is another postulated mechanism, albeit one that is challenging to objectively assess. Electron microscopic assessment of duodenal biopsies before and after 6 weeks of feeding a hydrolyzed protein diet to 20 dogs with CE identified significant reductions in mitochondrial lesion scores and reduced mean intermicrovillar spaces in the diet responsive dogs[52]. No differences in routine histopathology apart from reduction in mean mononuclear cell score in the LP were identified[52]. Thus, more sensitive techniques may be needed to illustrate the beneficial effects of diet.

Diet is a powerful modulator of gut microbial populations. Several studies have documented the effects of various diets on the gut microbiota of healthy and CE dogs[143,161,231,232]. In the studies of CE dogs, investigators have attributed clinical improvement to changes in gut microbial community makeup[42,162], which may or may not be valid. With current knowledge, it is impossible to predict which combination of intestinal microbes trigger positive or negative reactions in an individual host. Microbes tolerated by one host may be marked as foreign invaders by another. Data from massive studies of human microbiota in IBD and health show marked interindividual variability and temporal instability of the fecal microbiota in diseased patients[233]; this instability has also been appreciated in CE dogs[145]. Almost every study examining the fecal microbiome in dogs with CE makes its conclusions from a single time point. Regression toward the mean should be considered whenever investigators comment about a “reduced distance” toward the healthy population. This is not to rule out the possibility of microbiota-related disease remission with diet, more to emphasize that correlation is not causation, and microbiota studies with small sample sizes are at risk for over-interpretation.

Keeping these limitations in mind, how might diet-related microbiome shifts modulate disease activity? Studies in mice have established strong links between bacterial populations and development or loss of oral tolerance[234],[235]. This might underlie the apparent transient nature of CE in some dogs, if the intestinal immune system is 'retrained' to not react to commensal microbes or dietary components. This is supported by documentation of reductions in proportions of IgG and IgA coated bacteria decreased in CE dogs after hydrolyzed protein or limited antigen dietary intervention, in concordance with reduced disease severity. Of note, this reduction was not seen in the dogs treated with antibiotics or immunosuppressants[145].

Diet also may promote the expansion of microbes with essential functions within the gut lumen. For example, a small subset of the GI microbiota can convert primary to secondary BAs in the gut lumen via 7α-dehydroxylation[236]. Wang et. al. correlated hydrolyzed protein diet-related remission with recovery (elevation) of fecal secondary BA concentrations and shifts in fecal bacterial populations in 20 dogs with CE[42]. Likely, these mechanisms, and others that have not been described, work in concert to enable CE dogs to achieve clinical remission with nutritional intervention.

Other omics platforms may help clarify diet's impacts on the GI tract of CE dogs, adding practical consequences to microbiota shifts. Only a few studies have been completed to date. Feeding a fiber enriched diet to dogs with CE and large bowel diarrhea was associated with reductions in bacterial proteolytic processes along with significant reductions in fecal ammonium concentrations[43]. These changes may be influenced the limited protein content of the diet under investigation, particularly because they identified no differences in fermentative capacity of fecal microbes despite taxonomic shifts[237]. A study evaluating the impact of a hydrolyzed protein diet with or without a probiotic glycosaminoglycan supplement found that CE dogs post-diet (regardless of supplement) evidenced significant changes in the serum glycerophospholipid profile[238]. They postulated that restoration of intestinal epithelial cell structure and function could

underlie the diet's clinical benefit. Metabolomics therefore have potential to refine the complex impacts of diet on GI homeostasis in dogs with CE, but more well-designed studies are needed.

CE Dogs Without Response to Dietary Intervention

Despite the availability of more veterinary therapeutic diets (at least in the United States) than at any other point in the past, there remains a subset of CE dogs that do not achieve clinical remission with diet. Since long-term outcomes in non-diet responsive CE dogs are poor, this subset of patients is particularly of interest. Overall, 15 to 40% of CE dogs are non-responsive to all therapies implemented[3]. The typical approach to these patients is escalation of immunosuppressive therapies, which is not always associated with favorable outcomes. It is possible that subsets of dogs with unique disease processes exist within the CE umbrella which require distinct interventions. A prime example of this is the disease formerly known as "boxer colitis"[63]. Affected dogs suffered from severe colitis along with weight loss and anemia, and many died after initiation of immunosuppressive therapies or were euthanized due to refractory disease. Decades of investigation ultimately identified intramucosal *E. coli* as the causative agent, and now outcomes are much improved with therapy guided by antimicrobial susceptibility profiling[239]. In human medicine, Celiac disease provides an example of a unique form of IBD. Unravelling of its pathogenesis led to the key finding that a strict gluten-free diet, not immune suppression, is all that is needed for management[188]. Still, in *E. coli*-associated GC and Celiac, histological differences clearly distinguished these conditions as outliers compared to most patients presenting with GI symptoms. Intestinal biopsy histopathology has not proven beneficial to distinguish dogs with non-responsive CE, so other approaches need to be utilized.

1.4 Transcriptomic Investigation of Chronic Intestinal Disorders

Why Transcriptomics?

Transcriptomics is the study of messenger RNA (mRNA) molecules or transcripts, providing a read out of expressed genes at the time of sample collection. This method is an accessible means of specifically characterizing molecular processes in disease-relevant samples that is one step closer to physiology than what can be gleaned from genomic approaches. Initial transcriptomic investigations utilized hybridization-based approaches such as microarrays; these quantify expression levels of pre-selected genes within a single experiment. Microarray readouts suffered from imprecise gene expression quantification and poor reproducibility[240] and so have largely been replaced by RNA-sequencing (RNA-seq). This approach quantifies all expressed genes and provides a broad overview of cellular processes and pathways[241]. Sequencing of mRNA from endoscopically obtained biopsy samples results in a gene signature reflective of all the numerous cells that comprise the intestinal mucosa, a major advantage over histopathology where readily identifiable cells (e.g., inflammatory ones) are given preferential attention.

Transcriptomic Studies of Chronic Intestinal Disorders in Humans

Transcriptomics have advanced understanding of human IBDs through RNA-seq analysis of intestinal tissues. Haberman et. al. completed an early and thorough RNA-seq study of ileal samples from 359 pediatric CD patients, finding elevated expression of the antimicrobial peptide gene dual oxidase (DUOX2) and reduced expression of lipoprotein gene APOA1[242]. Incorporating expression levels of these 2 genes into a regression model improved accuracy in predicting remission at 6 months compared to clinical factors alone[242]. A more recent study of non-inflamed colonic biopsies from adult CD patients identified 2 distinct molecular subclasses, one of which exhibited genes more characteristic of ileal tissue[243]. When this approach was applied to samples from pediatric, treatment-naïve CD patients, a similar ileum-like group was identified, who were found to have an elevated risk of future colectomy due to refractory disease. Thus, transcriptomics offers the potential to highlight subgroups of patients with shared

molecular biology which would not be apparent with routine approaches. Bujko et. al. performed RNA-seq on macrophage subsets after FACS sorting. This revealed 5 distinct small intestinal macrophage populations, some of which were phenotypically like circulating macrophages[244]. Flow cytometry used in tandem with RNA-seq in their study, enabling substantial advancement of understanding of a key cell type in intestinal physiology. Concurrent RNA-seq and microbiota analysis of colonic biopsies from 13 human patients with cystic fibrosis (CF) compared to those from 12 healthy individuals identified differential expression of the chloride channel CFTR, but also enrichment in genes associated with tumor suppression, metastasis of colorectal cancer (CRC), and p53[100]. The mucosal microbiota was less diverse in CF patients versus healthy, and abundances of CRC-associated bacteria were significantly associated with expression of CRC-related genes including LCN2 and DUOX2[100]. These findings highlighted host-microbe interactions which could contribute to the earlier in life onset and more aggressive biological behavior of CRC seen in CF patients[245]. Despite only about a decade of widespread use of RNA-seq, this methodology has already contributed to substantial advancements in understanding of human IBD.

Investigation of Gene Expression in Canine CE

Transcriptomic investigation of CE in dogs is minimal. While numerous studies have been completed evaluating the expression of one or a handful of genes, none of these capture the breadth of a sequencing-based study. A few groups have investigated micro-RNA (miR) expression in serum, feces, and colonic biopsies samples. Konstantinidis et. al. found increased expression of miR-16, miR-21, mi-R122 and mi-R147 in the colon of 26 dogs with CIE showing clinical signs compatible with colitis[246]; each also showed increased relative expression in serum samples. Another study compared expression of 6 miR using digital droplet PCR (ddPCR) to see if those markers could distinguish inflammatory from neoplastic lesions[247]. The tumor suppressive miRs, miR-192 and mi-R194, showed lower expression in the lymphoma samples

compared to healthy, but levels were not different from levels in enteritis samples[247]. The utility of miR for diagnosing and monitoring CE in dogs, and their contributions to pathogenesis, remains unknown.

A single publication using a microarray approach compared duodenal gene expression in biopsies from 13 CE dogs compared to those from 5 PLE and 6 healthy dogs[34]. Over 1,800 genes were differentially expressed (DE) between all diseased (CE + PLE) dogs compared to healthy, with reduced relative expression in diseased samples in 85% of those genes. Markedly downregulated genes in diseased dogs included neurotensin, glucagon, and fatty acid binding protein 6; genes with increased relative expression in CE included RNase A family 1 and several S100 calcium-binding protein genes (S100G, S100A2, A100A10). Of note, when subgroup analysis was completed on genes with >3-fold expression differences, the most marked differences were between PLE dogs and healthy dogs, and only 1 gene, CXCL13, was significantly DE comparing severe CE (CIBDAI ≥9) dogs with normal albumin levels to healthy dogs[34]. All in all, the transcriptome of intestinal tissues from CE dogs has not been characterized. Comparison of transcriptomic profiles from healthy and CE dogs, as well as from CE dogs before and during treatment interventions, could be instrumental to expand understanding the pathogenesis of CE.

Limitations of Bulk RNA Sequencing Analysis

Bulk RNA-seq is not without its limitations. Firstly, RNA degradation can occur, which may preferentially impact certain genes[248]; this is particularly relevant for samples containing high levels of RNases such as pancreas and lung and stool[249]. Also, while it may be tempting to take transcriptomic results at face value and assume that gene expression is equivalent to cellular activity on a protein and metabolite level, there is no linear relationship between gene expression and protein content[250]. Individualized RNA variations may result in distinct proteomes[251]. Thus, validation studies are necessary to determine the consequences of

differentially expressed genes or implicated pathways. Larger RNA molecules must be fragmented to be compatible with most sequencing platforms, which may hamper library construction[252]. Transcriptome coverage must be weighed with cost per sample when determining sampling depth, otherwise limitations of microarrays may arise. Finally, it may be impossible to determine if gene expression differences are due to different proportions of cell types or levels of gene expression within individual cell types[253]; this distinction may lead to markedly different conclusions. Despite these obstacles, RNA-seq offers huge advantages in its ability to reflect complex physiology, accurately quantitate expressed genes, and avoid reliance on pre-existing knowledge and species-specific reagents[241].

Single Cell RNA Sequencing

Single cell RNA-seq (scRNA-seq) offers yet an even more detailed means of characterizing cellular identities and activities within heterogeneous tissues[254]. In the high throughput version of this approach, samples are processed to result in live single cell suspensions which are then individually tagged using microfluidics. After library preparation, sequences are determined and mapped back to the cell of origin. Since its invention in 2009, scRNA-seq has added to the body of knowledge on a diverse range of GI topics in mice and in humans. Through scRNA-seq analysis of ileal biopsy samples from inflamed and uninflamed regions of 12 Crohn's patients, Martin et. al. identified a key role for CCR2 ligand producing stromal cells in Crohn's disease pathogenesis[255]. Individuals with a certain collection of stromal cells and inflammatory macrophages showed refractoriness to anti-TNF therapy. Moreover, the investigators were able to identify this collection of cells within 4 separate bulk RNA-seq datasets and confirm their theory that the cell signature correlated with refractoriness TNF blockers. Therefore, scRNA-seq offers the opportunity to extend the scope of a study beyond the samples directly analyzed. This approach has also advanced understanding of development

of the intestinal immune system and regional differences along the length of the gut, of rare cell subtypes like tuft cells, and contributions of non-immune cells to GI homeostasis[254].

Drawbacks of scRNA-seq exist. This technology is not particularly good at resolving splice variants or antigen receptors. Assignment of cell identities can be challenging and relies upon the quality of the reference genome and iterative analyses of cell subtype gene expression. Automated tools can be massively helpful with this but have not been established for all tissues in all species. Processing of tissues can bias for certain cell types[256] and therefore impede accurate characterization the cellular make up of heterogeneous tissues. In this case, complementary techniques like flow cytometry and immunohistochemistry serve as important controls. As compared to bulk RNA-seq, scRNA-seq is much more labor intensive and expensive. However, once tissue-specific cell atlases are generated, deconvolution approaches can be utilized to obtain an estimation of cellular composition of tissues analyzed with bulk RNA-seq[257,258].

1.5 Conclusions

Chronic enteropathy in dogs is an exceedingly common condition negatively impacts the quality of life of dogs and their families. Traditional approaches, such as measuring the concentrations of individual biomarkers and analyzing intestinal biopsy histopathology and fecal microbial populations have not allowed for actionable advancements in its understanding. Practically, this results in the heterogeneous mix of patients under the broad CE umbrella being subjected to treatment trials that may or may not address their specific pathology. Repeating the same types of studies and analysis techniques have not allowed for practical advancements in CE understanding and disease management over the past 2 decades. New approaches, such as those employing next generation sequencing technologies, are needed to comprehensively characterize the canine gut both in health and in patients with CE. Initial signals need to be followed up to explore the mechanistic basis behind shifts in metabolites and gut bacterial

populations. Accomplishing these goals will result in meaningful findings that will allow for practical improvements in the diagnosis and management of CE in dogs, as well as harness the translational potential of these animals that enrich the lives of so many humans.

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Chapter 2: MODULATION OF CANINE MACROPHAGE RESPONSES IN VITRO BY PRIMARY AND SECONDARY BILE ACIDS[1]

2.1 Summary

Bile acids (BA) are important metabolites secreted into the intestinal lumen and impacted by luminal microbes and dietary intake. Prior studies in humans and rodents have shown that BAs are immunologically active, and that primary and secondary BAs have distinct immune properties. Therefore, the composition of the gut BA pool may influence GI inflammatory responses. The current study investigated the relative immune modulatory properties of primary (cholic acid, CA) and secondary BAs (lithocholic acid, LCA) by assessing their effects on canine macrophage cytokine secretion and BA receptor (TGR5) expression. In addition, RNA sequencing was used to further interrogate how CA and LCA differentially modulated macrophage responses to LPS. We found that exposure to either CA or LCA influenced LPS-induced cytokine production via macrophages similarly, with suppression of TNF-α secretion and enhancement of IL-10 secretion. Neither BA altered the expression of the BA receptor TGR5. Transcriptomic analysis revealed that CA activated inflammatory signaling pathways in macrophages involving type II interferon signaling and the aryl hydrocarbon receptor, whereas LCA activated pathways related to nitric oxide signaling and cell cycle regulation. Thus, we concluded that both primary and secondary BAs are active modulators of macrophage responses in dogs, with differential and shared effects evident with sequencing analysis.

[1] This chapter includes the complete published manuscript: Modulation of in vitro macrophage responses via primary and secondary bile acids in dogs by Manchester AC, Chow L, Wheat W, Dow S. Animals. 13, 3714.

2.2 Introduction

Chronic enteropathy (CE) in dogs is thought to be driven by chronic intestinal inflammation, yet the pathogenesis of the disease is incompletely understood. As potential regulators of gut immunity, intestinal bile acids (BAs) are increasingly recognized to be important signaling molecules within and outside of the gut [1]. Bile acids mediate their activity in part through the G-protein-coupled receptor TGR5, which is expressed by both macrophages and intestinal epithelial cells [2]. In mice and humans, TGR5 activation plays a role in BA synthesis, energy homeostasis, hepatic regeneration, and inflammatory responses [3, 4, 5]. The secondary BA lithocholic acid (LCA) is the strongest endogenous agonist of TGR5 [6], with lesser agonist activity exerted by the primary BAs cholic acid (CA) and chenodeoxycholic acid (CDCA). Studies in rat, rabbit, and human cells have highlighted the immunomodulatory role of BAs, with suppression of LPS-stimulated TNF-α production via macrophages exposed to LCA and CDCA but not CA[6,7,8,9].

Specific members of the intestinal microbiota perform critical BA transformation reactions, including 7α-dehydroxylation, which is necessary for primary BA conversion to secondary BAs[10]. Secondary BAs, including LCA, are the most abundant BAs in the distal gut lumen in health[11], but the balance of primary and secondary BAs may be disrupted in chronic intestinal diseases in humans (e.g., inflammatory bowel diseases, irritable bowel syndrome[12]) and in dogs[13,14]. These disruptions in BA pool makeup (specifically, expansion of primary BA relative to secondary BA) are associated with more severe intestinal inflammation[12,15,16] and dysbiotic excursions[13, 17], which may play a role in the pathogenesis of CE in dogs. While the overall impacts of these changes in BA levels are incompletely understood, evidence from a recent study found that clinical improvement in dogs with CE fed a hydrolyzed protein diet was associated with increased concentrations of secondary BAs [13]. These findings suggest that restoring normal intestinal luminal BA composition may be an important mechanism by which therapeutic diets induce clinical remission from CE.

As an example of the relevance of intestinal BA profiles, we and others reported that oral administration of certain antimicrobials commonly used to treat dogs with CE, including tylosin and metronidazole, has been associated with changes in intestinal BA pool, with a shift towards a BA profile dominated by primary BAs[18,19]. Dogs with CE treated with antibiotics typically have poor long-term responses and struggle with recurrent GI signs[20,21]. The fecal microbiota of dogs with CE is characterized by reduced relative abundances of Firmicutes and Clostridia and increases in the abundance of Proteobacteria[22,23,24]. These alterations in bacterial relative abundances may be important as a diminished prevalence of anaerobic bacteria such as *Clostridium* and *Bacteroides* spp. able to perform BA biotransformation reactions[25,26] is correlated with a reduction in proportions of fecal secondary BAs[14]. Restoring the normal distal gut BA profile may therefore be an important therapeutic endpoint.

In the study reported here, we investigated and compared differences in innate immune responses to primary and secondary BAs in dogs. The studies used a canine macrophage cell line and primary cultures of monocyte-derived macrophages to determine the impact of BAs on LPS-induced macrophage cytokine production, along with transcriptomic responses. The study revealed distinct differences in innate immune responses to primary versus secondary BAs in dogs and suggested that these two classes of BAs may be important factors in regulating intestinal immunity in health and disease.

2.3 Materials and methods

Biochemical Reagents

Bile acids (cholic acid (CA; C1129-25G); lithocholic acid (LCA; L6250-10G); sodium taurolithocholate (T-LCA; T7515-100MG); and chenodeoxcycholic acid (CDCA; C9377)) and sodium azide (S2002) were obtained from Sigma-Aldrich Co., St. Louis, MO, USA. Lipopolysaccharide from E. coli 055:B5 was sourced from InvivoGen, San Diego, CA, USA.

Human macrophage colony stimulating factor (M-CSF; cat#300-25-50UG) was obtained from PeproTech, Thermo Fisher Scientific, Waltham, MA, USA.

Cell Culture Medium

Cells were cultured in Dulbecco's modification of eagle's medium with 4.5 g/L glucose, L-glutamine, and sodium pyruvate (DMEM; Corning, Manassas, VA, USA) with the addition of 10% fetal bovine serum (FBS; Peak Serum, Wellington, CO, USA), CTM, and 2-mercaptoethanol (Gibco by Life Technologies Corporation, Grand Island, NY, USA) at 55 mM. Cells were expanded in 300 cm^3 vented-cap tissue culture flasks (Fisherbrand, Waltham, MA, USA) and plated in flat-bottom low-evaporation lid 24-well cell culture plates (Falcon, Corning Incorporated, Corning, NY, USA).

Canine Macrophage Cell Lines

Two canine macrophage tumor cell lines were used in these studies. One canine macrophage cell line (MH588, generated in the Dow lab) was isolated from a lymph node aspirate of a dog with malignant histiocytosis. The cell line was phenotyped using flow cytometry to confirm macrophage-like cell identity (expression of CD14 and CD11b; Supplemental Figure S1). A second canine macrophage cell line (DH82) was obtained from the American Type Tissue Collection (Gaithersburg, MD, USA) and has previously been extensively characterized and used in canine macrophage assays[27, 28, 29]. The MH588 and DH82 cells were cultured in complete medium (see above) using incubators at 37 °C with 10% CO_2. Cells were detached from flasks with trypsin for 10 min, resuspended in fresh medium, and seeded at 1×10^5 cells/well in 24 well plates for cytokine release and RNA sequencing assays. Cells were cultured to 80% confluency prior to the initiation of treatment.

Generation of Monocyte-Derived Macrophages

Monocyte-derived macrophages (MDM) were generated, as previously reported [30]. Briefly, peripheral blood mononuclear cells (PBMC) were prepared from EDTA-collected blood samples from healthy dogs (CSU IACUC #1440) and seeded at a density of 2–5 × 10^6 cells per ml in 48-well plates. After allowing cells to adhere for 4 h, the medium with non-adherent cells was gently removed and replaced with fresh DMEM containing M-CSF at 50 ng/mL. Cells were differentiated for 7 days, with media changes with M-CSF after 3 and 5 days. On day 7, the media was replaced, and cells were treated as noted.

Bile Acid Treatment of Macrophage Cultures

Triplicate wells of macrophages were treated with BAs at 25 μM; this concentration was selected to fall between estimates of BA concentrations in the intestinal lumen (<1000 to 10,000 μM), plasma BA concentrations (25 to 50 μM), and fecal BA concentrations in people (up to 5 mM)[31, 32]. After 2 h of incubation, cells were activated with lipopolysaccharide (LPS) at 100 ng/mL; this concentration was selected as a conservative extrapolation from studies of bacterial-derived LPS levels in the gut lumen and within inflamed gut mucosa[33,34,35]. After 48 h of bile acid and LPS co-culture, supernatants were harvested and frozen at −20°C until ELISA evaluation. The macrophage activation and cytokine release assays were repeated 6 times to ensure reproducibility. After supernatants were removed, cells were rinsed with PBS, then trypsinized for 10 min, and then resuspended in flow cytometry staining buffer (FACS) for flow cytometry (see below).

Flow Cytometry for Assessment of Macrophage Expression of the Bile Acid Receptor TGR5

Cells were collected following 48 h of stimulation with LPS ± BAs, and expression of the bile acid receptor TGR5 was measured using flow cytometry. The antibody used was a polyclonal

IgG rabbit anti-human TGR5 antibody (Abcam, Cambridge, UK). The secondary antibody used was donkey anti-rabbit polyclonal IgG Cy3-conjugated (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Negative controls included cells labeled with isotype-matched antibodies, including ChromePure Rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

To assess expression of TGR5, cells were incubated with anti-TGR5 antibody (diluted 1:100) together with 5% normal donkey serum (as a block for non-specific binding) for 30 min at 4°C. Cells were then washed, incubated with secondary antibody (donkey anti-rabbit IgG), washed, and then resuspended in PBS with FACS buffer (PBS with 2% FBS and 0.1% sodium azide) for analysis. To gate out dead cells, 2% 7-aminoactinomycin D (7-AAD; Thermo Fisher Scientific, Waltham, MA, USA) was added just before analysis.

Immunostained cells were analyzed using a Beckman Coulter Gallios flow cytometer (Brea, CA, USA). The analysis was performed using FlowJo software (Ashland, OR, USA). The analysis included the determination of the percentage of positive fluorescent cells in addition to the geometric mean fluorescence intensity of labeled cells. Background fluorescence was determined using either unstained cells or cells stained with isotype-matched antibodies.

ELISA Assays for Canine Cytokines

Cytokine concentrations were measured using canine-specific ELISA assays (DuoSet ELISA canine IL-10 [DY735] and TNF-α [DY1507], R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Validation has been performed by the company, and this ELISA has been used extensively by our group and others [36,37,38]. Cytokine concentrations were extrapolated from a standard curve generated with recombinant cytokines as part of the ELISA kit. Absorbance was measured at 450 nm using a Biotek plate reader.

Assessment of TGR5 Expression via Fluorescence Microscopy

Macrophages were cultured on cell culture chamber slides (In Vitro Scientific, Sunnyvale, CA) overnight, then fixed with 2% paraformaldehyde and immunostained with anti-TGR5 antibody resuspended in FACS buffer with 0.25% saponin for cell permeability, as described previously [30].

MTT Assay

Macrophages were cultured in a 96-well plate as described above prior to the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent (MTT) at 0.25 mg/mL to test for cell viability by assessing redox potential [39]. After 4 h at 37 C, 100 uL of MTT stop (HCl) was added, wells were triturated vigorously, and absorbance was read using a plate reader at 570 nm.

RNA Sequencing and Analysis Pipeline

The impact of BA treatment on macrophage transcriptomic responses was assessed using RNA sequencing as described by us previously [30, 40, 41]. Briefly, MH588 cells were pre-incubated with BA for 2 h prior to stimulation with LPS and then cultured for another 24 h, at which point RNA was extracted from cells using a Qiagen RNeasy mini kit (Qiagen). Extracted RNA was sent to Novogene Corp. (San Diego, CA, USA) for sequencing using an Illumina platform. RNA quality was determined using the Agilent 4200 Bioanalyzer system. The sequence library was generated using the NEBNext Ultra II RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA) following the manufacturer's instructions. Libraries were sequenced on an Illumina NovaSeq 6000 (Illumina, San Diego, CA, USA). Paired-end reads 150 bp in length were generated, and files were delivered as de-multiplexed fasq files. Analysis of RNA sequencing data was completed using Partek Flow software, version 10.0 (Partek Inc., Chesterfield, MO, USA). Raw reads were filtered for adapters and reads containing N > 10% and for Phred scores >30. Alignment was conducted using STAR 2.7.3a[42], and with the CanFam3.1 genome assembly, reads were annotated and counted using HT-seq with Ensembl 107[43].

Statistical Analysis

Basic statistical analysis was completed in GraphPad Prism for macOS (version 10.0.1). Differences between cytokine concentrations with different treatments were assessed with a one-way ANOVA with *P* values adjusted for multiple comparisons. Adjusted *P* values < 0.05 were considered statistically significant. Expression of the BA receptor TGR5 was assessed qualitatively via immunocytochemistry (ICC) and quantitatively via flow cytometry; the numbers of positive cells were compared using a one-way ANOVA. For RNAseq analysis, GSA (gene set analysis) (https://documentation.partek.com/display/FLOWDOC/GSA accessed 14 April 2023) was performed on normalized transcript counts, and differentially expressed genes were filtered using *P* values and fold change −1.5< or >1.5. The STRING database was used to assess inferred protein–protein interactions[44].

2.4 Results

Impact of Primary and Secondary BAs on Cytokine Secretion by LPS-Activated Canine MH588 Macrophages

The first question we addressed was how primary and secondary BAs may differentially impact cytokine secretion via TLR4-activated macrophages as a model for the modulation of immune responses via gut macrophages responding to TLR-activating stimuli such as LPS. To address this question, we used an in vitro model system (Figure 2.1) employing canine macrophages activated with LPS and co-incubated with two different primary BAs (CA and CDCA) and a free or taurine-conjugated secondary BA, LCA. For most of these studies, macrophages were pre-incubated with BAs for 2 h prior to LPS activation. However, in some experiments, the BAs were added simultaneously. We found the order of incubation did not affect overall cytokine responses. Previous studies with human macrophages have demonstrated that taurine-conjugated LCA suppresses pro-inflammatory cytokine production and gene expression via LPS-

activated macrophages, supporting the generation of a more regulatory or immune-suppressive phenotype [9, 45].

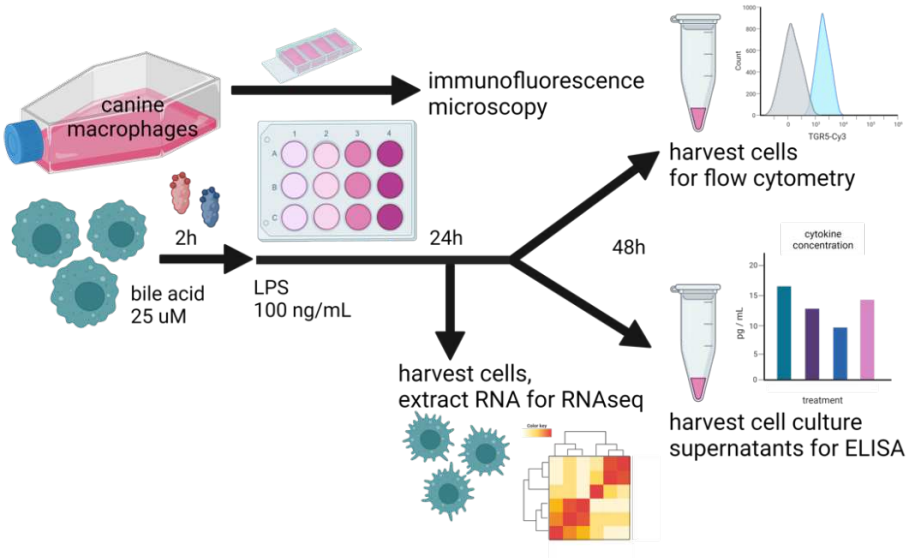


Figure 2.1. Overall study design, whereby cultured canine macrophages (MH588 cells) were plated in a 24-well plate, incubated with bile acid at 25 μM, and stimulated with LPS 2 h later. After 48 h of co-culture, cells were immunostained for flow cytometry, and supernatants were harvested for cytokine analysis. For the RNAseq portion of the analysis, cells were harvested for RNA extraction after 24 h of co-culture.

We observed first that incubation with primary or secondary BAs alone did not activate MH588 macrophages (data not shown); specifically, neither release of TNF-α nor IL-10 was measurable in culture supernatants after 48 h of incubation with any of the 4 BAs screened. Therefore, LPS-activated macrophages were used for the rest of the studies reported here because LPS is a key immune-activating molecule to which macrophages in the GI tract are continuously exposed. When BAs were added to LPS-activated MH588 macrophages, cytokine production was modulated differently by primary vs. secondary BAs. For these studies, we measured a key pro-inflammatory cytokine (TNF-α) previously shown to be upregulated in intestinal biopsies of dogs and humans with enterocolitis[46, 47]. Macrophage production of a key immune modulatory/suppressive cytokine (IL-10) was also measured to assess the potential immune suppressive properties of BAs.

We then determined whether there were concentration-dependent effects of BAs on LPS-stimulated TNF-α release via macrophages (Supplemental figure 2.2), based on estimated physiological concentrations of BAs in the intestinal lumen[32] and dose titration experiments in our canine macrophage system (data not shown). In addition, we assessed the impact of BAs on macrophage viability using an MTT assay and found no impact at the concentrations tested, including 25 μM (data not shown). Therefore, we selected 25 μM as the concentration that was used in the studies reported here. In initial screening experiments with three unconjugated BAs, we found that the most marked differences on cytokine release were observed between CA and LCA (Supplemental figure 2.2) following LPS activation, so these BAs were selected for subsequent studies. DH82 cells failed to produce either TNF-α or IL-10 following LPS stimulation (data not shown), whereas MH588 cells produced strong TNF-α and IL-10 responses. Therefore, MH588 cells were used for the remainder of the studies shown here.

Incubation of LPS-activated MH588 macrophages with the primary BA CA did not alter macrophage secretion of TNF-α compared to LPS alone. The other primary BA, CDCA, induced a mild reduction in TNF-α release. We next examined the impact of LCA on macrophage cytokine responses following LPS activation. We found that treatment with the secondary BA LCA significantly suppressed TNF-α production and also increased the anti-inflammatory cytokine IL-10 release (Figure 2.2). Taurine-conjugated LCA (TLCA) suppressed TNF-α release but failed to stimulate IL-10 release (Figure 2.2B). These findings indicated that, like other species, the secondary BA LCA was immunologically active and significantly suppressed LPS-stimulated macrophage inflammatory responses. The primary BAs varied in their immunosuppressive qualities, with CDCA acting to suppress TNF-α release but neither CDCA nor CA modulating IL-10 release.

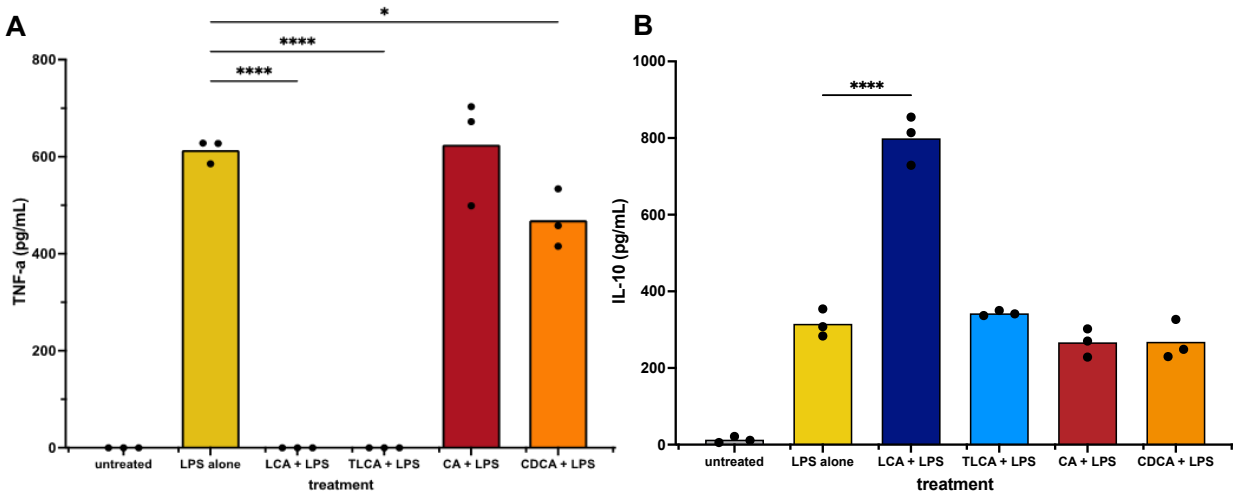


Figure 2.2. TNF-α (A) and IL-10 (B) concentrations in supernatants from canine macrophages (MH588) treated with bile acid at 25 μM, incubated for 2 h, then stimulated with LPS at 100 ng/mL. Values were compared with one-way ANOVA with the Holm–Sidak multiple comparison test. Horizontal bars with asterisks indicate significantly different mean values compared to control treatment of LPS alone; * $p < 0.05$ and **** $p < 0.0001$.

Impact of Primary and Secondary BAs on Cytokine Secretion by LPS-Activated Primary Monocyte-Derived Macrophages

We next assessed the impact of BAs on cytokine secretion by primary cultures of canine macrophages to ascertain whether they responded differently to BAs than the macrophage cell line. We found that the addition of LCA significantly suppressed TNF-α production via LPS-activated MDMs, which was consistent with responses by MH588 cells (Figure 2.3). In addition, LCA treatment also augmented the production of IL-10 from activated MDM, again in agreement with the results from the MH588 experiments (Figure 2.2). Alternatively, we found that CA suppressed TNF-α secretion via LPS-activated MDMs, suggesting that CA may have immune suppressive properties in dogs.

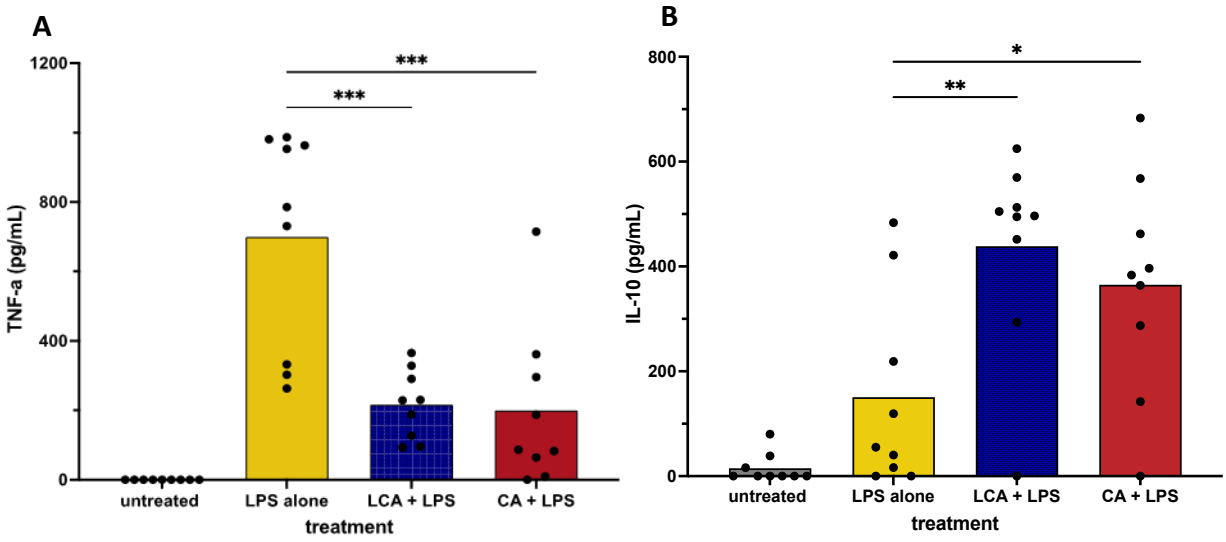


Figure 2.3. TNF-α (A) and IL-10 (B) concentrations in supernatants from monocyte-derived macrophages from healthy dogs treated with bile acid at 25 □M, incubated for 2 h, then stimulated with LPS at 100 ng/mL. Values were compared with one-way ANOVA with the Holm–Sidak multiple comparison test. Horizontal bars with asterisks indicate significantly different mean values compared to control treatment of LPS alone; *** $p < 0.0001$.

Impact of BA Exposure on TGR5 Expression

Bile acids signal to intestinal and immune cells via several receptors, particularly the receptor TGR5 [1]. Therefore, we assessed whether MH588 cells expressed TGR5 and whether exposure to BAs modulated the expression of TGR5. We found that TGR5 was highly expressed on canine MH588 cells (Figure 2.4). However, exposure to CA or LCA did not alter TGR5 expression, thus indicating that altered receptor expression would be unlikely to account for any observed differences between primary and secondary BAs and their effects on macrophage immune responses (Supplemental figure 2.3).

Macrophage Transcriptomic Responses to BAs

To interrogate the impact of CA and LCA more fully on canine macrophage function following LPS activation, we performed RNA sequencing (RNAseq) on MH588 cells that were stimulated for 24 h with LPS (100 ng/mL) alone or together with either CA or LCA at 25 μM.

We observed that treatment with CA prior to LPS activation significantly altered the macrophage transcriptomic response to this stimulus and resulted in the greatest number of

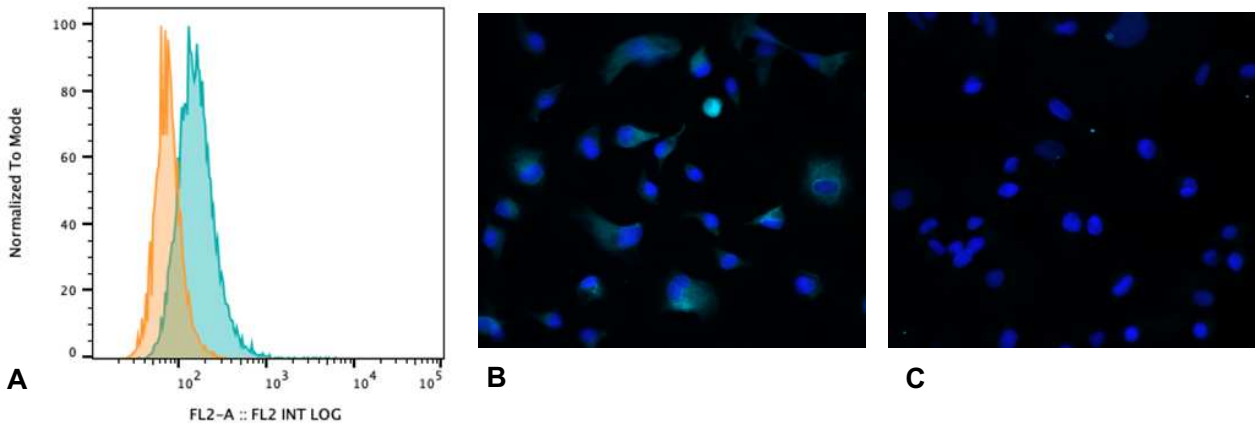
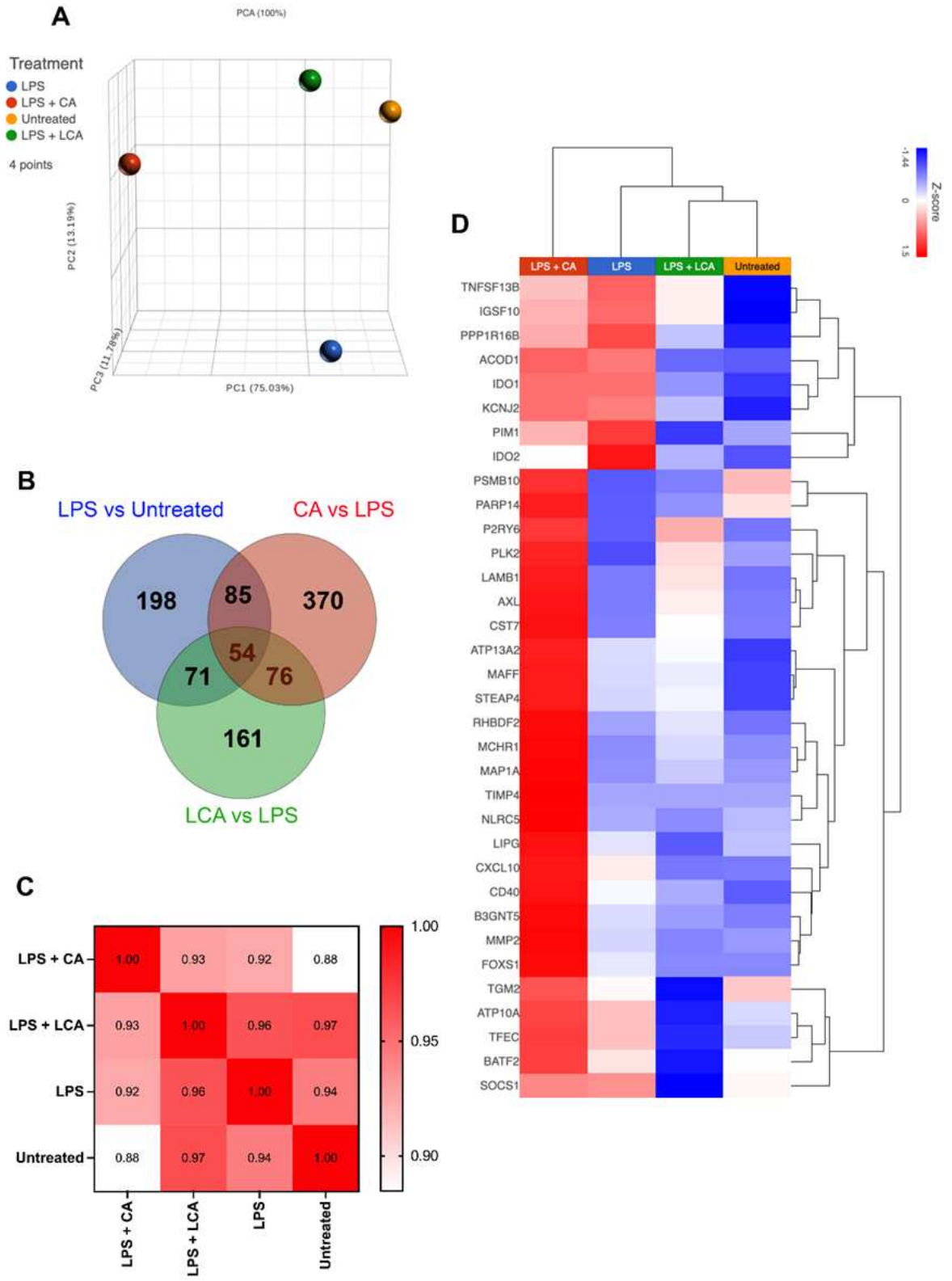


Figure 2.4. (**A**) Cell surface TGR5 expression by MH588 cells as assessed using flow cytometry. The different shadings represent different stains; orange is isotype control (rabbit IgG), and green is antibody of interest. (**B**) Fluorescence micrographs of MH588 cells at 20× magnification with un-conjugated anti-TGR5 antibodies (1:50 dilution) compared to (**C**) staining with rabbit isotype control.

differentially expressed genes (DEGs) compared to cells treated with LPS alone or with LPS followed by LCA (Figure 2.5A-C). LPS activation of the MH588 cells triggered activation of many genes associated with immune response pathways, many of which we have described recently for canine macrophages[30] (Figure 2.5D). Specifically, we found that CA treatment further upregulated expression of many genes associated with activated macrophages (so-called M1 macrophages), including C-X-C motif chemokine ligand 10 (CXCL10), matrix metallopeptidase 2 (MMP2), and MAF bZIP transcription factor F (MAFF). Treatment with CA was associated with downregulation of Ras homolog family member H (RHOH), IL18R1, prostaglandin E synthase (PTGES), and nuclear receptor subfamily 5 group A member 2 (NR5A2).

In contrast, macrophage treatment with LCA was generally inhibitory to the expression of M1-associated pro-inflammatory and chemokine genes (CXCL10, MAFF), in addition to those involved with cytokine signaling (SOCS1) and extracellular matrix remodeling (MMP2; Figure 2.5D). Treatment with LCA following LPS activation was associated with enhanced expression of TNF-ASF13B (role in B cell activation), P2RY6, and AXL receptor tyrosine kinase (AXL), genes that are associated with anti-inflammatory effects through distinct pathways. However, LCA treatment was associated with decreased expression of IDO1 and IDO2, which would be expected to have a pro-inflammatory effect.


A
PCA (100%)
Treatment
LPS
LPS + CA
Untreated
LPS + LCA
4 points
PC2 (13.19%)
PC3 (11.78%)
PC1 (75.03%)
B
LPS vs Untreated
CA vs LPS
LCA vs LPS
198
85
370
54
71
76
161
C
LPS + CA
LPS + LCA
LPS
Untreated
1.00
0.93
0.92
0.88
0.93
1.00
0.96
0.97
0.92
0.96
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1.00
0.95
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D
LPS + CA
LPS
LPS + LCA
Untreated
TNFSF13B
IGSF10
PPP1R16B
ACOD1
IDO1
KCNJ2
PIM1
IDO2
PSMB10
PARP14
P2RY6
PLK2
LAMB1
AXL
CST7
ATP13A2
MAFF
STEAP4
RHBDF2
MCHR1
MAP1A
TIMP4
NLRC5
LIPG
CXCL10
CD40
B3GNT5
MMP2
FOXS1
TGM2
ATP10A
TFEC
BATF2
SOCS1
Z-score
-1.44
0
1.5

Figure 2.5. Transcriptomic analysis of LPS-activated macrophages treated with CA or LCA. Macrophages were incubated with LPS and BAs for 24 h, then RNA was extracted for RNAseq, as described in Methods. (**A**) Principal component analysis plot showing differences in transcriptome by distance. (**B**) Venn diagram showing overlapping gene sets using differentially expressed genes defined with an arbitrarily determined *p* value of 0.05. (**C**) Pearson correlation of similarities between samples. (**D**) Heat map hierarchical clustering of M1 genes upregulated in CA compared to LCA and untreated. Gene set was extrapolated from 250 M1 genes defined in Chow et al.'s 2022 study of *in vitro* differentiated canine macrophages[30].

We also examined and compared the impact of treatment with CA versus LCA on individual macrophage-expressed genes (Table 2.1, Supplemental figure 2.4) and biological pathways (Figure 2.6) in LPS-activated macrophages beyond those considered to be related to M1 macrophages. Significantly upregulated genes in CA-treated, LPS-activated macrophages compared to LCA-treated macrophages include C-C chemokine motif 8 (CCL8), interleukin-1α (IL-1A), interleukin-1β (IL-1B), and C-X-C chemokine motif 6 (CXCL6). Downregulated genes associated with CA treatment when compared to LCA-treated macrophages included Src-like adapter (SLA), Fas apoptotic inhibitory molecule 3 (FCMR), and P-selectin glycoprotein ligand 1 (SELPLG) (Table 2.1). Significantly upregulated genes in LCA-treated, LPS-activated macrophages included IL18R1, prostaglandin E synthase (PTGES), and NR5A2 (Supplemental figure 2.4).

Pathway analysis using gene set enrichment analysis (GSEA) identified key immune pathways associated with macrophage responses to CA or LCA. This analysis revealed that treatment with CA upregulated inflammatory response pathways in LPS-activated macrophages involving the aryl hydrocarbon receptor, Type 2 interferon signaling pathways, and non-genomic actions of vitamin D3 (Figure 2.6). Pathways enhanced via LCA relative to CA exposure included nitric oxide and cell cycle regulation (E2F, G2M) pathways.

Table 2.1 Summary of results comparing top 10 genes differentially regulated through exposure of canine macrophages to cholic acid (CA) compared to lithocholic acid (LCA) prior to LPS stimulation based on gene-specific analysis.

Gene ID	Description	Log2(Ratio) (LPS + CA vs LPS + LCA)
CCL8	C-C motif chemokine 8	4.13
RSAD2	Radical S-adenosyl methionine domain-containing protein 2	4.10
GAP43	Neuromodulin	3.96
IL1B	Interleukin-1 beta	3.37
IL1A	Interleukin-1 alpha	3.31
ENSCAFG00000016245	Adhesion G protein-coupled receptor E2	3.10
CXCL6	C-X-C motif chemokine 6	2.75
TBX3	T-box transcription factor TBX3	2.69
SERPINB2	Plasminogen activator inhibitor 2	2.62
TIFAB	TRAF-interacting protein with FHA domain-containing protein B	2.61
SELENOM	Selenoprotein M	2.61
ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase family member 2	2.45
FBP1	Fructose-1,6-bisphosphatase 1	- 6.34
PGF	Placenta growth factor	- 5.26
SLA	Src-like-adapter	- 5.18
FCMR	Fas apoptotic inhibitory molecule 3	- 4.61
CFAP45	Cilia- and flagella-associated protein 45	- 4.52
CRLF1	Cytokine receptor-like factor 1	- 4.47
SLC1A7	Excitatory amino acid transporter 5	- 4.40
KEL	Kell blood group glycoprotein	- 4.31
SAMD11	Sterile alpha motif domain-containing protein 11	- 4.26
KCNMA1	Calcium-activated potassium channel subunit alpha-1	- 4.25
TMEM59L	Transmembrane protein 59-like	- 3.76
SELPLG	P-selectin glycoprotein ligand 1	-3.74

Given the unanticipated similarities in LPS-stimulated cytokine production by the MDMs treated with CA or LCA, we examined the 130 genes whose expression was shifted in similar directions by these two BAs in the MH588 macrophages (Figure 2.5B). Protein–protein interactions showed nodes centered around innate immune genes (IL1B, IL1A, and MMP), cell-cycle-related genes (KIF4, TOP2A, and NCAPG), as well as integrins (ITGB3, ITGAV, and ITGA4;

Supplemental figure 2.5). In keeping with this, shared KEGG pathways included Toll-like receptor and IL-17 signaling, and GO processes included negative regulation of macrophage-derived foam

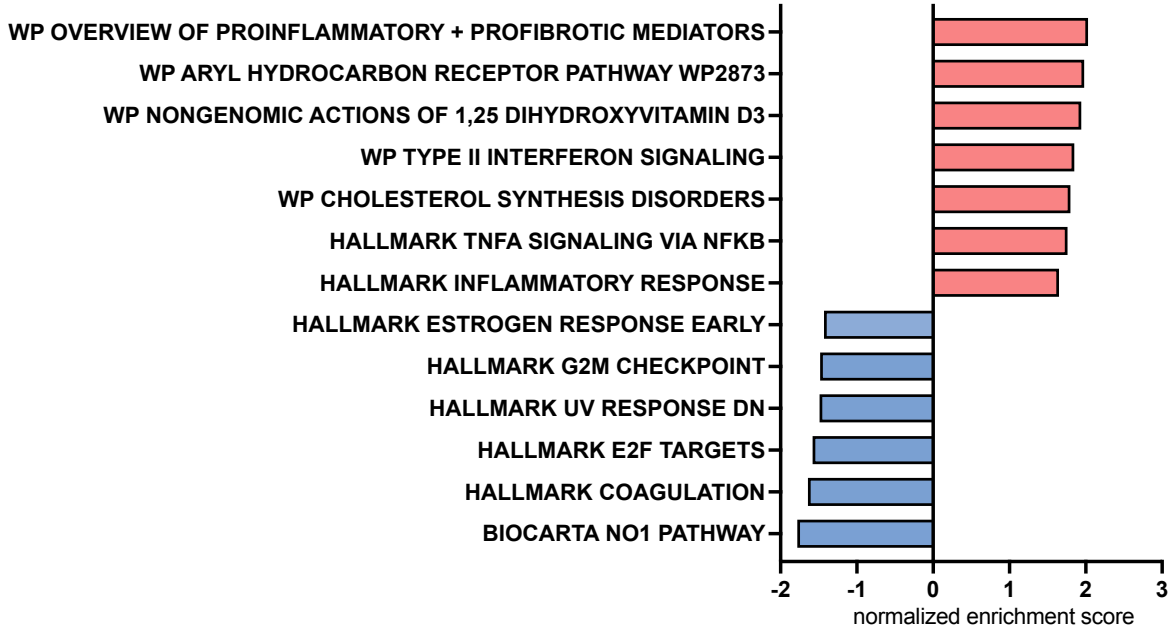

Figure 2.6. Bar chart representing pathway analysis results based on Gene Set Enrichment Analysis comparing canine macrophages exposed to CA (25 μM) to those exposed to LCA (25 μM) 2 h prior to stimulation with LPS (100 ng/mL). Red bars indicate pathways upregulated by CA compared to LCA, and green bars indicate pathways downregulated by CA compared to LCA. All displayed pathways were significantly differentially expressed with an FDR-adjusted p value < 0.2.

cell differentiation, low-density lipoprotein receptor activity, and lipoprotein metabolic processes. Thus, both CA and LCA appear to modulate immune responses and metabolic activities, in keeping with their far-reaching signaling capacities.

2.5 Discussion

Bile acids are known to play key roles in the absorption of lipids in the GI tract, but less is known regarding their immune modulatory roles. This study is the first to our knowledge to explore the immune modulatory functions of primary and secondary BAs in dogs. To address these questions, we used both canine macrophage cell lines and primary cultures of dog MDM to

examine the impact of key primary (cholic acid, CA) and secondary (lithocholic acid, LCA) bile acids on macrophage activation via LPS, to which gut macrophages are continuously exposed.

Immunomodulatory impacts of BAs have been described in cells from other species but not previously in canine macrophages. Our analysis produced mixed results, with indications of differential immune modulation by primary and secondary BAs in the MH588 cells, as in other species [6, 48]. In contrast, MDMs exposed to CA or LCA exhibited reduced TNF-α and enhanced IL-10 release compared to LPS alone. We noted, however, that LCA suppressed canine macrophage inflammatory responses, including significant suppression of LPS-stimulated TNF-α production, together with significant stimulation of IL-10 secretion. Thus, canine macrophages appeared to respond to LCA as expected based on studies with human, rabbit, and rat macrophages [3, 6, 7, 45]. This finding indicates that secondary BAs are likely to serve an important immune modulatory role in the canine GI tract.

For the current study, in addition to cytokine analysis with conventional ELISA and measurement of cell surface expression of macrophage activation markers, we also employed RNAseq to further interrogate the full breadth of BA effects on macrophage function. The transcriptome analysis of CA-treated macrophages indicated upregulation of key inflammatory genes (e.g., CCL8 and CXCL6) and pathways (e.g., aryl hydrocarbon receptor and type II IFN signaling). There was an indication of upregulation of TNF-α-related pathways by CA compared to LCA, which was not reflected by the cytokine concentrations in the MDM experiments. In addition, we found that CA increased expression of some inflammatory genes but failed to show significant upregulation of key genes such as those regulating TNF-α, IL-6, and IP-10 that are associated with macrophage inflammatory responses. Pathway analysis also highlighted the role of CA in stimulating genes related to the aryl hydrocarbon receptor. This receptor has key roles in tempering intestinal inflammatory responses as well as promoting antimicrobial peptide release[49], therefore providing more evidence of nuanced immune responses induced by the primary BA. Meanwhile, some features of LCA responses often countered those stimulated by LPS

(suppression of innate immune genes IL1A, IL1B, and CXCL8). However, LCA was not completely immune suppressive, as, for example, we observed upregulation of the nitric oxide pathway and upregulation of indoleamine-related genes (IDO1 and IDO2). Overall, LCA appeared to be the more important BA signaling molecule in tempering LPS stimulation in these studies, which agrees with its known higher affinity for TGR5 binding compared to other BAs [6].

These transcriptomic signatures of macrophage responses to BAs thus provided important new insights into mechanisms by which BAs modulate the function of gut immune cells, extending beyond commonly studied cytokines. The canine and human transcriptomic responses to BAs by macrophages were also compared to help assess the relative relatedness of the two species. A previous study by Wammers et al. utilized a microarray platform to evaluate the impact of taurine-conjugated LCA on human primary macrophages stimulated with LPS [9]. They found that out of 865 LPS-induced transcripts, 202 were significantly modified by taurine-conjugated LCA (TLCA), with the expression of 111 genes downregulated and the expression of 50 genes further upregulated[9]. Their study identified upregulation of matrix metallopeptidase genes (MMP10, MMP12) as well as downregulation of innate immune cell activating (IL1A, CCL4) and chemokine genes (CXCL1, CXCL9, CXCL10, CXCL11, IL18) when LPS-stimulated cells were exposed to TLCA. At the protein level, they identified suppression of TNF-α release by TLCA[9]. In concordance with the findings from human secondary BA immune assays, we also found that the secondary BA LCA curtailed LPS-stimulated pro-inflammatory and chemokine gene expression in canine macrophages. Moreover, NR5A2 (also known as LRH-1) was identified as one of the most significantly upregulated genes by LCA. This gene encodes for an orphan nuclear receptor with anti-inflammatory roles and impacts on cholesterol homeostasis and bile acid synthesis[50]. Thus, multiple pathways are likely to account for the diverse impacts of BAs. Given that BA dysmetabolism and dysbiosis have been observed with chronic inflammatory intestinal disorders in both species [14, 15], further investigation is warranted to examine the role of luminal BA in these patients and in dogs with CE.

Our findings suggest that there may be a plausible link between the microbiome, the metabolome, the diet, and immune responses in the gut in dogs, mediated at least in part by primary and secondary BAs. The study by Wang et al.[13] provided evidence that achievement of clinical remission through feeding of a hydrolyzed protein diet to dogs with CE correlated with increased concentrations of secondary BAs (LCA and deoxycholic acid), while concentrations of secondary BAs did not increase during dietary therapy in non-responders[13]. This study also identified the bacteriostatic properties of LCA against potential pathobionts, *E. coli* and *Clostridium perfringens*, which are associated with CE dysbiosis[51]. Our findings bolster a potential mechanistic link between the BA and microbiome shifts with dietary intervention in this population of dogs.

Beyond immune modulation, transcriptomic analysis of the MH588 macrophages pointed to impacts on metabolic activities common to CA and LCA. Recent proteomic and metabolomic studies have identified aberrant serum lipid profiles in CE dogs compared to healthy controls[52, 53]. Another group found that after a trial with a hydrolyzed protein diet, CE dogs had significant alterations in serum lipid levels[54]. Bile acids could influence disease manifestation from this direction as well.

TGR5 has been previously identified using immunohistochemistry and mRNA in situ hybridization on canine cells, including macrophages, enterocytes, and enteroendocrine cells [2]. Our results are important in that they demonstrate that regulation of TGR5 expression by BAs is unlikely to account for the differential effects of CA and LCA. However, it is possible that differential binding and signaling through TGR5 (and other BA receptors) could explain how CA and LCA exert their effects on immune cells in dogs. Lithocholic acid has been reported to be among the most potent agonists of TGR5 [6], and this might explain why canine macrophages responded more robustly to lower concentrations of LCA versus CA and CDCA.

Potential Impact of the New Findings

The studies reported here provide a mechanistic basis for understanding why the absolute concentration of secondary BAs can help determine the character of immune responses in the gut in dogs with CE. Our findings indicate that the primary BA, CA, in dogs appears to have both immune suppressive and immune stimulatory properties. Thus, the absolute concentration of the secondary BA LCA may be the more important parameter to measure to assess, for example, the impact of BA dysmetabolism on regulating GI inflammation in canine CE and other conditions. It remains to be seen below what threshold concentration the anti-inflammatory effects of LCA are lost.

Diet remains the most effective therapy for canine CE[55], and the ability of diet to alter microbial populations[56] and shifts in luminal BA populations may explain in part the impact of specific GI disease-targeted dietary interventions[13]. Antibiotics are another widely implemented treatment modality for canine CE[21, 57], and one possible mechanism for their efficacy in managing CE is through shifting the microbiome and, in turn, the BA-gut axis[57]. Counterintuitively, antibiotics commonly prescribed to dogs with diarrhea, like tylosin and metronidazole, are associated with the same disruption in fecal BA documented in CE dogs[18,19]. This may contribute to the lack of robust remission and raise questions regarding the long-term ramifications of this form of treatment. The model we describe here provides an in vitro system to further explore the connections between gut microbes, BA concentrations and ratios, and gut immune regulation and dysregulation.

Study Potential Weaknesses and How to Address Them

The experimental model used in these studies necessarily markedly simplified the complex gut immune milieu and focused on only a single cell type (macrophages) rather than on the full spectrum of gut mucosal cells. Exposure to different concentrations of BAs, as well as combinations of BAs, could have also influenced our conclusions and are important future experiments to complete to better represent the complex milieu of the intestinal lumen. The model

also did not use intestinal macrophages but instead relied on a macrophage cell line and on primary cultures of monocyte-derived macrophages. Nonetheless, the model allowed for careful dissection of immune responses to two of the most prevalent BAs in the intestinal lumen and can serve for further investigations of microbial and dietary impacts on gut immune responses. The model could potentially be improved by using macrophages isolated and purified from GI biopsies, though that system too would also be imperfect due to disruption of macrophage cell functions as well as local stromal cell connections imposed during tissue digestion. The use of intestinal organoids and the incorporation of macrophages into the multi-cell epithelial structures could also be used to model BA immune responses, including more intestinal mucosal cell populations, particularly epithelial cells. The results of the RNAseq studies, particularly for the expression of individual genes but also for confirming the presence of proteins, would need to be determined with RT-PCR, immunohistochemistry, ELISA, or targeted proteomics if possible. Nonetheless, the pathway analyses performed, which incorporate the impacts of multiple different genes, provided new insights that could not be replicated by measurement of individual gene expression or protein concentration.

2.6 Conclusions

In summary, our studies are the first to report that prevalent primary and secondary BAs, CA and LCA, in dogs elicit different innate immune responses, a finding that has significant implications for linking the gut metabolome to the gut immunome. By understanding the roles of primary and secondary BAs in modulating gut innate immune responses in dogs, we now have a model to help interpret the results of BA analysis from canine fecal samples in dogs with CE.

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CHAPTER 3: EFFICACY OF AN ELEMENTAL DIET IN ACHIEVING CLINICAL REMISSION IN DOGS WITH CHRONIC ENTEROPATHY[2]

3.1 Summary

Diet may induce clinical remission in dogs with chronic enteropathy (CE). Elemental diets (EDs), providing protein as amino acids, modulate intestinal immunity and microbiome in rodents and humans. The aim of this study was to evaluate the impact of an amino acid-based kibble (EL) on CE clinical activity and gastrointestinal (GI)-relevant variables. Client-owned dogs (n = 23) with inadequately controlled CE were included in this prospective, uncontrolled clinical trial. Diagnostic evaluation including upper and lower GI endoscopy was performed before study entry. Canine chronic enteropathy clinical activity index (CCECAI), serum biomarkers, and fecal microbiome were evaluated before and after 2 weeks of EL. Dogs with stable or improved CE remained in the study for another 6 weeks. Pre- and post-EL clinical and microbiological variables were compared statistically using a mixed model. After 2 weeks of EL, 15 of 22 dogs (68%; 95% confidence interval [CI], 47%- 84%) consuming the diet were classified as responders with a median (range) decrease in CCECAI from 6 (3-12) to 2 (0-9; P < .001). Fourteen of 15 responders and 2/7 nonresponders at 2 weeks completed the trial; all 16 were experiencing adequate control at week 8 with a median CCECAI of 2 (0-3). In total, 16/23 dogs (70%; 95% CI, 49%-84%) were responders. Feeding EL caused shifts in fecal bacterial communities, which differed between responders and nonresponders. Serum biomarker concentrations were unchanged throughout the study apart from serum alkaline phosphatase activity. Exclusive feeding of EL improved clinical signs in 16 of 23 dogs with uncontrolled CE. Fecal microbiome shifts were associated with response to diet and may represent a mechanism for clinical improvement.

[2] This chapter includes the complete published manuscript: Manchester AC, Steven Dow, Lyndah Chow, et. al. Efficacy of an elemental diet in achieving clinical remission in dogs with chronic enteropathy. *J Vet Intern Med*. 2023; 37(6):2322-2233. This article was reproduced with permission from John Wiley and Sons, Inc., Hoboken, NJ.

3.2 Introduction

Chronic enteropathy (CE) is a common condition in dogs characterized by persistent clinical signs referrable to gastrointestinal (GI) dysfunction such as diarrhea, vomiting, and poor appetite. Dietary therapy is a mainstay of CE management in dogs, with resolution of clinical signs reported in up to 69% of dogs after dietary intervention [1, 2, 3, 4]. Treatment success has been documented with hydrolyzed protein, limited ingredient, and fat-restricted diets [3, 5]. The mechanisms underlying clinical improvement after diet change are incompletely understood, but likely involve host, microbiome, and dietary factors. Compared to other strategies such as antibiotics and immunosuppressive drugs, dietary therapy has been associated with more robust, longer lasting periods of clinical remission in dogs with CE [6].

Food components are 1 of the many environmental factors that may incite and perpetuate CE in dogs [7]; effects may be direct, indirect, or both. Elemental diets (EDs) provide protein in the form of individual amino acids rather than as polypeptides (hydrolyzed diets) or intact protein [8, 9]. Carbohydrate, fat, vitamins, and minerals are added to make a complete and balanced diet. Nutrition provided in this manner is intended to be non-immunogenic and easily assimilated. Clinically, EDs are utilized during exclusive enteral nutrition (EEN) in human patients, whereby liquid formulations are administered to meet nutritional needs10. Pediatric Crohn's disease (CD) patients treated with EEN experience decreased disease activity, mucosal healing, and improved growth. The mechanisms underlying these improvements are incompletely understood [8,9,10]. Chronic enteropathy in dogs and CD in human are not analogous but appear to share disruptions in host/microbiome interactions [11] and intestinal epithelial barrier structure and function [12,13]. Dietary therapy for CE mimics EEN in that a single diet is provided exclusively for a defined period of time. In affected dogs, clinical improvement may be associated with inclusion or exclusion of certain ingredients, altered nutrient profiles (e.g., lower fat), improved digestibility, or other factors [14,15].

Given the potential for adverse reactions against diet and intestinal microbiota as well fat intolerance in CE dogs, we conducted a clinical trial with 23 dogs with inadequately-controlled CE to determine the impact of a novel diet for dogs deriving protein from individual aminos acids (Purina® ProPlan® Veterinary Diets EL, Nestlé Purina, St Louis, MO, USA; EL) on clinical and biochemical disease manifestations, including the fecal microbiome. Our primary hypothesis was that clinical remission would be achieved in at least 60% of dogs with CE willing to eat EL for at least 2 weeks; clinical remission from CE also was assessed at 8 weeks in dogs remaining in the study.

3.3 Materials & Methods

Case Selection Criteria

The primary study investigator (ACM) directly oversaw enrollment into this Colorado State University Institutional Animal Care and Use Committee (protocol # 1440) approved study. Dogs presented to Colorado State University's James L. Voss Veterinary Teaching Hospital (CSU-VTH) for increased defecation or decreased fecal consistency, vomiting, or decreased appetite with or without weight loss, regurgitation or flatulence were considered for entry into the study. Owners of eligible dogs reported inadequate control of CE signs, answering no to the question "is your dog currently experiencing adequate relief from its CE signs?" [16]. Clinical signs were present persistently for ≥3 weeks or intermittently for ≥6 months, despite interventions including dietary changes, antibiotics, immunomodulatory treatments, and other treatments. Each owner also completed a canine chronic enteropathy clinical activity index (CCECAI) survey [17], and to be included in the study the cumulative score needed to be ≥3.

Dietary Trial

The clinical trial schedule is presented in Figure 3.1. At the initial visit (T0), after obtaining informed written consent, dogs underwent a thorough diagnostic evaluation to exclude

specific infectious, structural, and metabolic causes for CE clinical signs. Exclusion criteria included diagnosis of hypoadrenocorticism or exocrine pancreatic insufficiency, GI anatomic abnormalities that could cause CE signs, severe hypoalbuminemia (≤ 2 g/dL), azotemia (serum creatinine concentrations >2 mg/dL), and clinically relevant anemia (Hct <30%). After the initial study visit, all dogs were administered a 5-day course of PO fenbendazole (50 mg/kg). Previously prescribed medications that did not appear to be necessary were discontinued. Medications deemed essential based on owner and primary investigator discussions were continued at a constant dose throughout the study.

After the initial visit, dogs were enrolled in the study and returned within 3 weeks for the baseline (T1) visit and diagnostic evaluation (Figure 3.1). At each visit, owners again were asked about adequate relief. They provided a fecal sample from their dogs (naturally passed within 12 hours of the appointment and kept at refrigerator temperature until arrival at the hospital) at this and all subsequent study visits. The primary investigator scored the fecal sample using Purina's fecal scoring chart (https://www.proplanveterinarydiets.ca/sites/g/files/2021-02/180107_PPPVD-Fecal-Scoring-Chart-UPDATE-EN-FINAL.pdf). Dogs were fasted for at least 12 hours before each visit. Upper and lower GI flexible endoscopy was completed in routine fashion at T1 by the primary investigator (ACM) in 22/23 dogs enrolled in the study (endoscopy was not performed in 1 dog because of the owner's risk aversion).

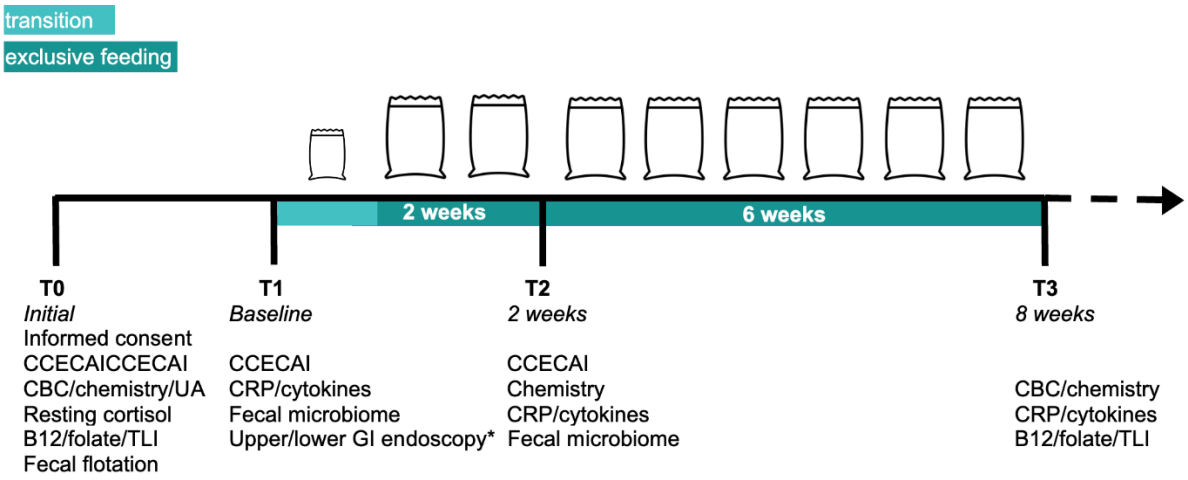

Figure 3.1. Schematic of study design involving feeding Purina® ProPlan® Veterinary Diets EL as single change for achievement of clinical remission of chronic enteropathy. Dogs were fasted for at least 12 hours prior to each study visit. *Upper and lower GI endoscopy were offered but not required for study participation. Endoscopy was consented to by 22/23 owners. Abbreviations: CCECAI, canine chronic enteropathy clinical activity index; CBC, complete blood count; UA, urinalysis; TLI, trypsin like immunoreactivity; CRP, C-reactive protein.

During the procedure, ≥10 biopsy samples were collected from the duodenum and ileum and ≥6 samples were collected from the stomach and colon. Samples were preserved in formalin and submitted to the Colorado State University Veterinary Diagnostic Laboratory for routine histopathological analysis; dogs were excluded if findings included anything other than inflammation without an identifiable inciting cause (i.e., infectious agent). Dogs with hypocobalaminemia (<300 ng/L) identified at the initial visit received cyanocobalamin SC once at the T1 visit (250 to 1000 mg depending on body weight). Owners were provided with an adequate supply of EL in unbranded packaging, along with specific feeding instructions, an 8-ounce measuring cup, and a study diary in which they noted the amount of kibble consumed, fecal scores, and any other observations.

Owners were instructed to gradually transition their dog to EL over 5 to 7 days. For dogs with normal or elevated BCS, the recommended daily allocation of EL was extrapolated from the dog's current caloric intake with the goal of weight maintenance. This amount was matched with an appropriate volume of EL (357 kCal/cup). In dogs with body condition score (BCS) <4/9, an

ideal body weight estimate[18] was used to calculate a recommended daily caloric intake. After 2 weeks of exclusive EL feeding, the owner returned with their dog and another fecal sample (identical guidance as T1 sample) for physical examination and blood draw (T2; Figure 3.1). For dogs whose CE was considered stable or improved compared to T1, another 6-week supply of EL was provided with instructions to continue feeding the diet exclusively. Throughout the study, owners had the option to remove their dog if clinical response was insufficient. The primary investigator removed dogs from the study if they failed to ingest ≥60% of calculated caloric needs for 2 consecutive days or had unacceptably severe CE signs.

At the end of 8 weeks of exclusive EL feeding (T3 visit), the owner returned with their dog and a final fecal sample. Repeat physical examination and study diagnostics were performed (Figure 3.1) and study participation concluded.

Serum Biomarker Assessment

Serum chemistry values were evaluated at the CSU-VTH Clinical Pathology Laboratory. Serum C-reactive protein (CRP) was measured in serum samples using the Gentian particle-enhanced turbidometric immunoassay as previously described [15] (Texas A&M Gastrointestinal Laboratory, College Station, TX, USA). The lower limit of quantification was 9.9 mg/L.

Serum cytokines were measured with the commercially available canine cytokine magnetic bead panel (MILLIPLEX, MilliporeSigma, Burlington, MA, USA) according to the manufacturer's instructions. Measured cytokines included GM-CSF, IFNg, KC-like, IP-10, IL-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, MCP-1, and TNF. Values are reported and compared in terms of mean fluorescence intensity (MFI).

Sample Handling

After analysis of the chemistry panel, remnant sera were collected from the CSU-VTH Clinical Pathology Laboratory and aliquoted and frozen at -80°C until analysis. Fecal samples

provided by owners or collected during study visits were stored at 4°C prior to aliquoting and freezing at -80°C.

Fecal DNA Extraction

Frozen fecal samples were thawed on ice and DNA was extracted from a 100 mg aliquot using Qiagen's PowerSoil Pro kit. DNA was eluted in 200 mL of nuclease free water to optimize concentration for downstream analyses. DNA quantity and purity were assessed by NanoDrop One spectrophotometer (Thermo Scientific, Waltham, MA) and samples were stored at -80°C.

Illumina 16S sequencing for bacterial ribosomal DNA

Frozen fecal bacterial DNA samples were sequenced by Novogene Inc. (Sacramento, CA, USA). Twenty-five base pair paired end V3/V4 sequencing was done on the Illumina NovoSeq 6000. Microbial sequencing and analysis were performed as previously described[19]. Reads with Qscore ≤5 or N>10 or length less than 60 bp were removed. Sequence analyses were performed with QIIME2 software[19]. Microbial community similarity was displayed with principal coordinate analysis (PCoA) plots. Alpha diversity was determined using Shannon, Faith, and Pielou indices. Beta diversity on weighted and unweighted UniFrac, Bray Curtis, and Jaccard measures were calculated in QIIME2. Alpha diversity indices were compared using a paired T-test, and β-diversity metrics were compared with PERMANOVA. Analysis of composition of microbiomes (ANCOM) was employed to determine the individual amplicon sequence variants (ASV) that differed significantly between time points within the responder (R) and non-responder (NR) groups[20].

Outcome Assessments

The primary outcome was subjective response to EL after 2 and 8 weeks of exclusive feeding; this was determined by the CCECAI score and the owner's impression of adequate

relief of their dog's CE clinical signs. If owners asserted that their dog was experiencing adequate relief from its CE and their CCECAI score decreased by >50% or to a value ≤ 3, the dog was classified as a R. Dogs not meeting both of those criteria were considered NRs. If outcomes at the 2 time points were discordant, the 8 week response was used to determine overall outcome. Secondary outcome measures included changes in biochemistry parameters and C-reactive protein concentrations, as well as alterations in the fecal microbiome after 2 weeks of EL feeding.

Statistical Analysis

Body weight values, clinical activity scores, and serum biomarker concentrations were assessed for normality using visual inspection of histograms and QQ plots. Data was generally skewed, and so numerical data was reported as median and range. Categorical data was reported in terms of frequency and proportion along with 95% confidence intervals. Differences in parameters before and during the exclusive feeding period were compared with either a two-way repeated measures ANOVA (no missing values) or a mixed model (missing values) between Rs and NRs at T2. Fixed effects included subject, treatment, time, and treatment-by-time interaction. For comparison of serum cytokine concentrations, MFI values were compared using a mixed model. P-values < .05, adjusted for multiple comparisons with Sidak's test, were considered significant. All statistical analysis was completed using Prism 9 for macOS.

3.4 Results

Patient Study Population

Twenty eight dogs with inadequately controlled CE were screened for enrollment. Reasons for exclusion included hypoalbuminemia (<2.0 g/dL), hookworm infection, exocrine pancreatic insufficiency, perianal fistulae, and chronic hypertrophic pyloric gastropathy in 1 dog each. The median age of the 23 dogs enrolled was 3.5 years (range 0.9-11) with a median

weight of 29 kg (4.4-65) and BCS 4/9 (2-7). Neutered males made up 78% (17/23) of the group, 5 were spayed females and 1 was an intact male. The most common breed was mixed (n = 7) followed by German Shepherd (n=4), Great Dane (n=2) and 1 each: miniature Schnauzer, English Setter, Staffordshire terrier, standard poodle, Weimaraner, Australian shepherd, Boykin spaniel, Labrador Retriever, Boston terrier, and Belgian Malinois. Duration of clinical signs prior to study enrollment ranged from 5 to 72 months (median 14). The most common clinical sign was abnormal defecation frequency or fecal consistency (22/23; 96%) followed by vomiting in 13/23 (57%) and poor appetite in 11/23 (48%). Median fecal score was 4 (2 to 7). Seventeen dogs had small bowel diarrhea and 4 had mixed bowel diarrhea. No dogs had isolated signs of large bowel diarrhea. Owners of 7/23 (30%) dogs reported weight loss or difficulty maintaining weight despite a good appetite. All dogs' CE had failed to improve with a diet change (including commercial well pet, veterinary therapeutic, and/or home-prepared diets); eighteen of 23 (78%) dogs had failed a trial with a veterinary therapeutic GI diet (limited ingredient, highly digestible, hydrolyzed, or high fiber; Supplemental table 3.1). This included 9/18 (50%) dogs that had previously failed a 14 day trial with ≥1 hydrolyzed protein diet.

Comorbidities in the study population included anxiety (n = 8), atopic dermatitis (n = 6), excess body weight (n = 3), and 1 dog each with atrial septal defect, dilated cardiomyopathy, chin furunculosis, osteoarthritis, cutaneous lupus erythematosus, and dermal endocrine cysts. All dogs had serum albumin concentrations > 2 g/dL, though 3/23 were below the hospital's canine reference range (3 g/dL). Two dogs had baseline cholesterol concentrations below the reference interval. Six of 23 dogs had serum cobalamin concentrations <300 ng/L and 1/23 had hypofolatemia. Fecal flotation identified parasitism at T0 in 2/19 dogs (1 *Giardia* and 1 *Monienzia* spp.). Giardia was absent after fenbendazole treatment and the Monienzia was ignored. All dogs had abdominal ultrasound performed within 6 weeks of enrollment and none were found to have GI tract structural abnormalities or abdominal masses.

Gastroduodenoscopy and ileocolonoscopy with biopsies were performed in 22/23 dogs (1 owner refused given risk aversion). The duodenum was biopsied in all dogs and the ileum in 18/22. Duodenal biopsy findings with hematoxylin and eosin staining included lymphoplasmacytic enteritis with or without an eosinophilic component in 21/22 study dogs. The other dog had primarily plasmacytic enteritis. Evidence of duodenal lacteal dilation was noted in 10/22 (45%) dogs. Details of histopathological findings are reported in Supplemental table 3.2. Due to excessive numbers of intraepithelial lymphocytes in duodenal biopsies from 2 dogs, the polymerase antigen receptor rearrangement (PARR) assay was submitted to CSU-VTH Clinical Hematopathology Laboratory. Veterinary Diagnostic Laboratory. A polyclonal rearrangement was documented in the immunoglobulin and T cell receptor genes in 1 dog. The other dog had a "possibly clonal" T cell receptor gene PARR result. This dog was enrolled nonetheless given the absence of weight loss, anemia, intestinal muscularis thickening and biopsy architecture distortion suggestive of small cell lymphoma.

Eight out of 23 dogs were continued on medications or supplements throughout the study period. For details, see Supplemental table 3.3. One dog was restarted on metronidazole (8.5 mg/kg PO q12h) following endoscopy but before introduction of EL due to intractable diarrhea. The 6 dogs with a cobalamin concentration <300 ng/L were administered a single dose of cyanocobalamin (250 to 1000 mcg depending on weight) subcutaneously at the T1 visit. No dogs received additional cobalamin supplementation before study exit. This deviation from commonly recommended protocols[21] was intended to put dogs at a level playing field at the time of diet initiation.

Clinical Responses

All but 1 of the 23 study dogs ate greater than or equal to the recommended volume of EL during the initial 2 weeks. One dog (#4) refused to eat EL; he was considered a treatment failure and excluded from further analysis.

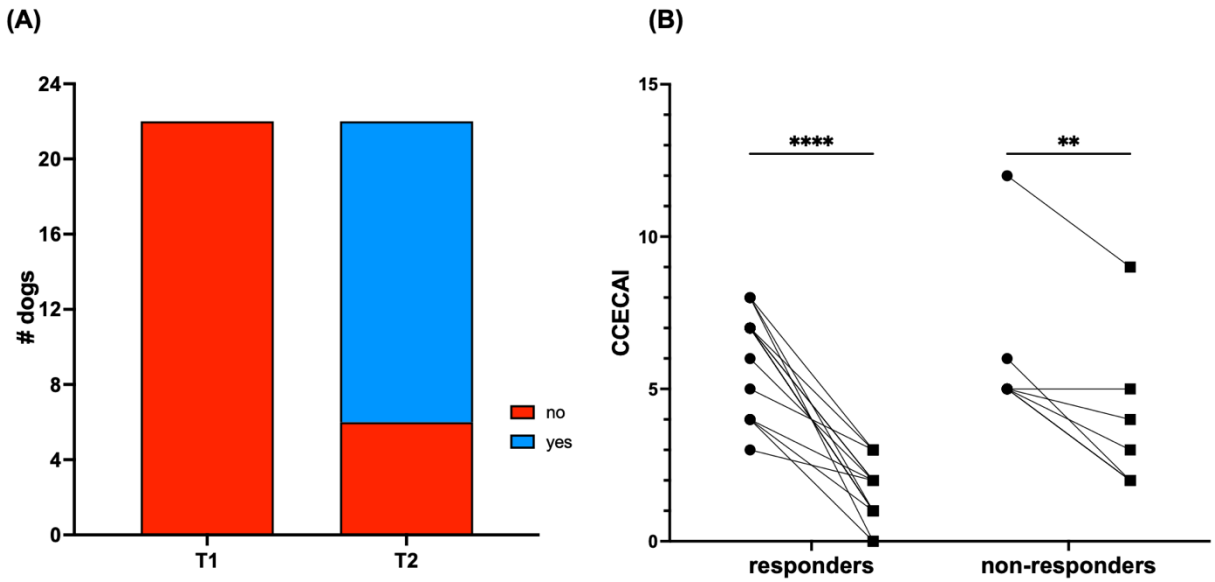

Figure 3.2. (**A**) Stacked bar graph representing owners' responses to the question "is your dog currently experiencing adequate relief from their chronic enteropathy signs?" before (T1) and after 2 weeks of EL feeding in 22 dogs ingesting EL. (**B**) CCECAI scores based on owner's assessment of 22 dogs before (circles) and after (squares) 2 weeks of EL feeding. Dogs with >50% reduction in CCECAI score or CCECAI < 3 and whose owners responded "yes" to the adequate relief question were considered responders (n=15) at T2; dogs not fulfilling both criteria were considered non-responders (n=7). Values compared with a two-way repeated measures ANOVA with *P* values adjusted for multiple comparisons. Significant differences indicated by horizontal line with asterisks above.

Our primary endpoint was the impact of EL on CE clinical signs. After 2 weeks of EL feeding, there were no significant differences in BCS or body weight. Owners of 16/22 (73%; 95% CI 52-87) dogs felt their pet was experiencing adequate relief from CE (Figure 3.2). Individual dog (P=.008), time (P <.0001), and the interaction of time with response to treatment (P=.01) impacted CCECAI score at T2, with a significant decrease in both R and NR dogs (Figure 3.2). A CCECAI ≤3 or >50% reduction occurred in 17/22 dogs (77%; 95% CI 57-90). Considering these measures together, at T2, 15/22 (68%; 95% CI 47-84) dogs were Rs and 7/22 (32%; 95% CI 16-53) were NRs. Median T2 Purina fecal score assessed by the supervising clinician was 2 (range: 1-7). The proportion of dogs with normal fecal consistency based on owner's response to the CCECAI survey question increased from 1/22 (5%; 95% CI 0-22 to 16/22 (73%; 95% CI 52-87) after 2 weeks of EL; owners of 8 dogs noted firm, dry feces.

Owners of 15/15 T2 Rs and 6/7 T2 NRs consented to continued study participation. One owner elected exit at T2 due to the dog's insatiable appetite, persistent flatulence, and coprophagia (#22; T2 NR). Within 11 days of the T2 visit, 4 additional dogs (all T2 NRs) exited the study. All exited due to unacceptable CE signs including unformed feces in 3 dogs (#s 2, 3, 17) and worsened pruritis and very firm, dry feces in 1 dog (#7). A fifth dog (#16; T2 R) developed a fibrocartilaginous embolus resulting in acute paraparesis after 5 weeks of EL feeding. The dog was started on oral prednisolone (0.67 mg/kg/d) and so was removed from the study at that point. Results from these 6 dogs are truncated at T2 visit.

The remaining 16 dogs returned after approximately 8 weeks of EL feeding (T3). Neither body weight nor BCS were significantly different compared to T1. While 6/6 dogs assessed to have a suboptimal BCS at T1 gained weight (median 6%, range 4-19), none achieved their goal weight calculated from initial BCS18. Median fecal score at T3 was 2 (range 1-4). Feces were deemed normal consistency by the owner in 11/16 dogs (69%; 95% CI 44-86). Of the 16 dogs completing the 8 week trial, all 16 were experiencing adequate relief from their CE and median CCECAI was 2 (range, 0-3). Using the combined criteria, the overall clinical response rate was 70% (16/23; 95% CI 49-84); two dogs classified as T2 NRs were Rs at T3.

Serum Biomarkers

No significant differences were noted in serum albumin after 2 weeks of EL feeding (Figure 3.3A). Median cholesterol was higher at T2 (204.5 mg/dL, range 105-306) compared to before EL (197.5 mg/dL, range 110-461) with a significant impact of time; this difference was only significant for Rs (Figure 3.3B; P = .01). Regardless of R or NR status, median serum ALK-PHOS activity was higher at T2 (68.5 U/L, range 26-281; Figure 3.3C) compared to before EL (41.5 U/L, range 10-142; P .037).

Serum CRP was below the lower limit of detection in 39/42 samples and was not significantly different in available sera from 21 dogs at T2 compared to before EL. None of the

serum cytokine MFIs were significantly different comparing these two time points, regardless of clinical response status (data not shown).

After 8 weeks of EL feeding, all 16 Rs dogs had B12 concentrations >300 ng/L (median 578; range, 338-1000) despite no supplementation during that period (5/16 dogs received single SQ dose at T1). Serum folate concentrations in the Rs were significantly higher at T3 (med 18.2 mg/L, range 13.8-27.1) compared to before the diet (med 13.1, range 5.1-18.5). Neither serum CRP concentrations nor cytokine MFIs were significantly different in samples from 15 Rs after 8 weeks of EL feeding compared to pre-EL (Supplemental figure 3.1).

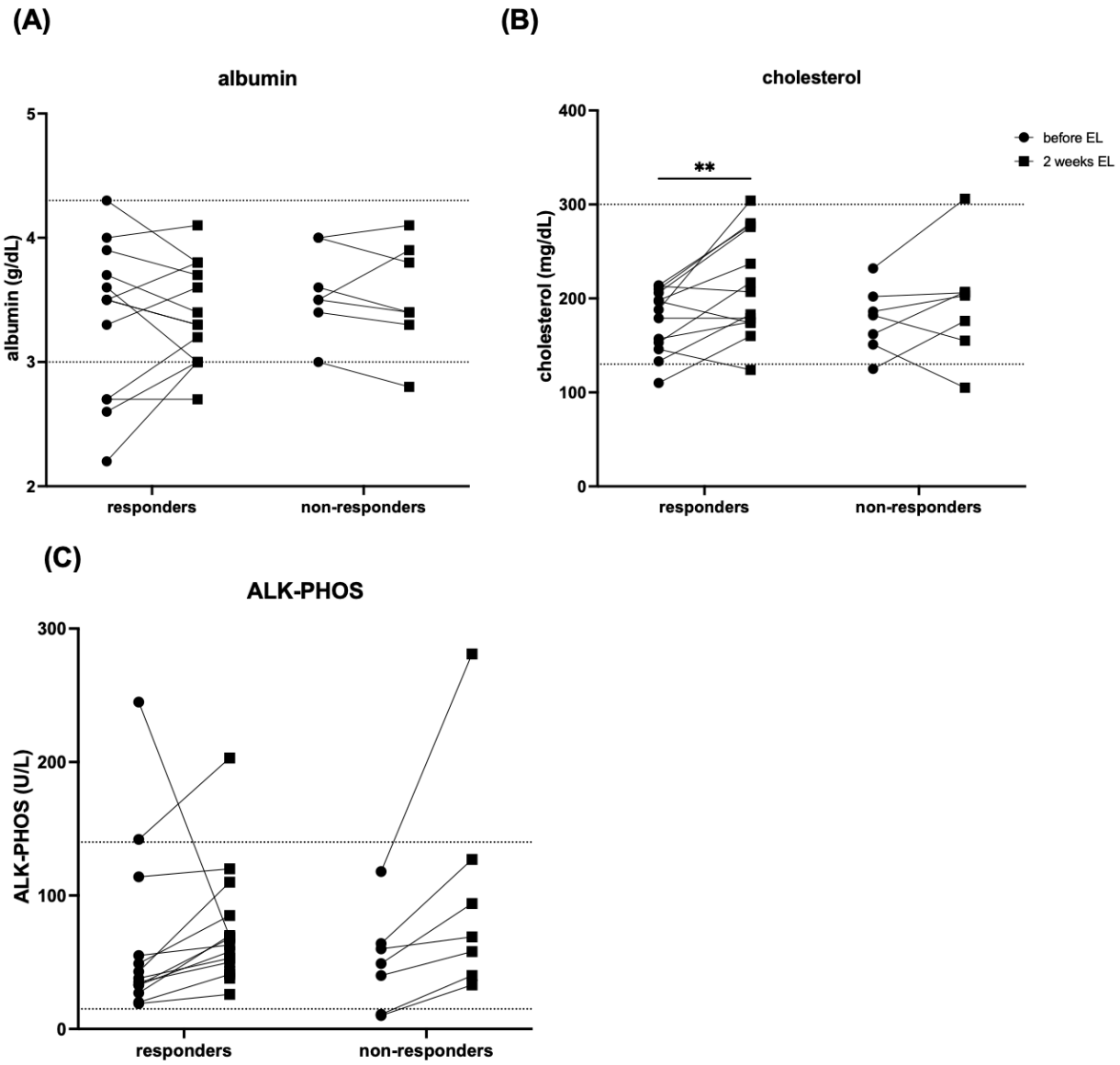

Figure 3.3. Serum (**A**) albumin, (**B**) cholesterol, and (**C**) alkaline phosphatase before (n=22) and after 2 (n=20) weeks of exclusive EL feeding, divided based on clinical response at T2. Circles represent dogs before the EL diet and squares represent dogs after 2 weeks of EL. Dotted lines denote the reference range. Values were compared with a mixed model followed by Šidák's multiple comparisons test with *P* values < .05 deemed significant and demarcated with a horizontal line and asterisks. Abbreviation: EL, Purina® ProPlan® Veterinary Diets EL; ALK-PHOS, alkaline phosphatase.

Fecal Microbiome

We concurrently investigated the impact of EL feeding on the fecal microbiome, using16S rRNA gene sequencing on available fecal samples from 18/22 study dogs before and after 2 weeks of EL feeding. Samples from 4 dogs were not included in the analysis due to lack of sufficient fecal material in either the T1 or T2 sample in 3 dogs (2 Rs: #13, 23; 1 NR: #22) and

initiation of metronidazole between T1 and T2 in 1 dog (NR; #17). The predominant bacterial phyla present in feces before EL were, in order of decreasing relative abundance, Firmicutes, Fusobacteria, Proteobacteria, Bacteroides, and Actinobacteria. This same relative ranking of bacterial prevalence was present after 2 weeks of EL. There was no significant difference in alpha diversity (Faith, Pielou's evenness or Shannon) in fecal microbiota before EL versus T2 regardless of clinical response (Supplemental table 3.4).

Principle coordinate analysis allowed visualization of the similarities between fecal microbial community compositions before and at T2 (Figure 3.4). All the similarity metrics

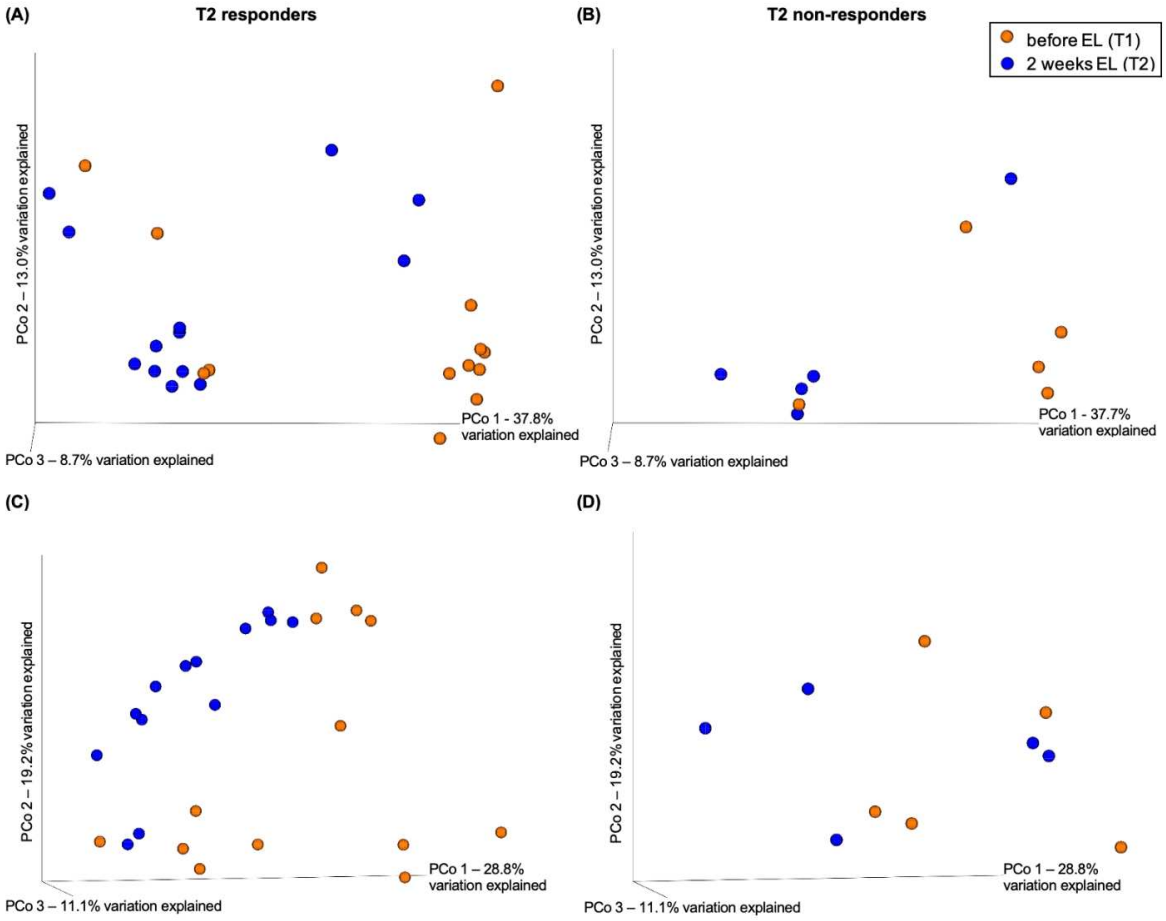

Figure 3.4. Principal coordinates analysis generated using uniform number of randomly subsampled reads and either unweighted UniFrac distances (**A** & **B**) or weighted UniFrac distances (**C** & **D**), showing similarity of fecal microbial communities from dogs before (T1; orange circles) and after 2 weeks of EL feeding (T2; blue circles). Panels A & C display data from dogs classified as responders at T2, and panels B & D display data from T2 non-responders. Dogs with ≥50% reduction in CCECAI score or CCECAI < 3 and whose owners responded "yes" to the adequate relief question were considered responders (n=13) at T2; dogs not fulfilling both criteria were considered non-responders (n=5). Abbreviations: PCo, principal coordinate.

(unweighted and weighted UniFrac, Bray Curtis, Jaccard) revealed heterogeneity, with overlap between the before and T2 samples. PERMANOVA testing identified significant differences in b-diversity comparing before diet to T2 samples in the 13 Rs that were not present in the 5 NRs (Table 3.1; Figure 3.5).

Table 3.1. Results of PERMANOVA comparison of beta-diversity indices in 18 dogs before and after 2 weeks of EL feeding (T2) and based on clinical response (n=13 responders and n=5 non-responders) at that time point.

	responders		**non-responders**	
similarity	***P* value**	**pseudo-F statistic**	***P* value**	**pseudo-F statistic**
Unweighted UniFrac	.01	4.364	.18	1.749
Weighted UniFrac	.01	2.98	.66	0.732
Bray Curtis	.01	2.108	.58	0.844
Jaccard	.01	2.618	.12	1.443

To uncover the ASVs contributing to changes in bacterial communities, ANCOM was applied to compare pre-diet to T2 samples within the R and NR groups. Results highlighted a differential impact of EL feeding on the fecal microbiome of Rs compared to NRs (Figure 3.5). The three significant differences in NRs were a reduction in the phylum Tenericutes, the class *Mollicutes* and the order *Anaeroplasmatales* (Table 3.2). Within the Rs, significant changes in relative abundances were seen at the phylum, class, order, family, and genus levels; these included reduced relative abundances of *Bifidobacteriaceae, Lactobacillaceae, Streptococcaceae* and *Desulfovibrionales* (Table 3.2).

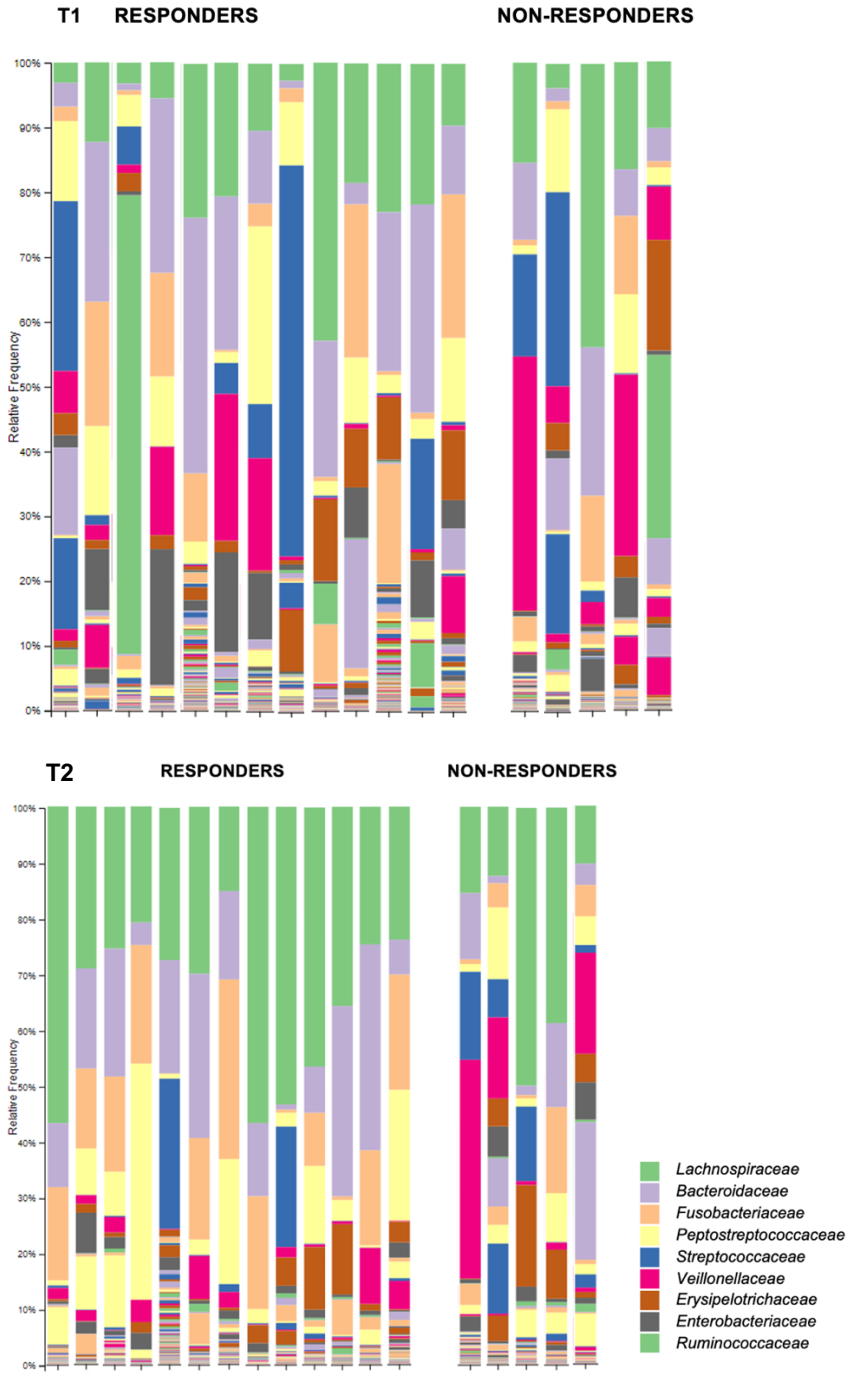

Figure 3.5. Stacked bar chart depicting most abundant bacterial families in CE dog fecal samples (n=18) before (T1) and after 2 weeks of EL feeding (T2) based on 16S sequencing. Bars are grouped based on responder (n=13) vs. non-responder (n=5) status at that time point; responders were dogs whose CCECAI score decreased by ≥50% or to a value <3 and whose owners answered "yes" to the question, "is your dog currently experiencing adequate relief from its chronic enteropathy?". Representative colors are repeated. The legend indicates the bacterial family associated with the first (starting at top) appearance of that color.

Table 3.2. Amplicon sequence variants identified as differentially abundant before and after 2 weeks of EL feeding based on ANCOM analysis within the responders (n=13) and non-responders (n=5). Abbreviations: R, responder; NR, non-responder; ASV, amplicon sequence variant.

T2 responders	Level	ASV	T2 vs. T1	W value	F statistic
	Phylum	*Nitrospirae*	increase	4	1.53
		Tenericutes	decrease	3	-1.77
		Firmicutes	decrease	2	-0.8
		Verrucomicrobia	increase	2	1.08
	Class	*Deltaproteobacteria*	decrease	10	2.58
		[Spartobacteria]	increase	7	-1.45
		Bacilli	decrease	4	1.98
		Nitrospira	increase	4	-1.9
	Order	*Bifidobacteriales*	decrease	17	3.36
		[Chthoniobacterales]	increase	11	-1.53
		Desulfovibrionales	decrease	11	2.5
		Lactobacillales	decrease	8	2.51
	Family	*Bifidobacteriaceae*	decrease	15	-3.23
		Lactobacillaceae	decrease	14	-3
		S24-7	decrease	9	-2.71
		Desulfovibrionaceae	decrease	9	-2.37
		Streptococcaceae	decrease	8	-2.81
	Genus	*Bifidobacterium*	decrease	14	-3.16
		S24-7 unnamed genus	decrease	12	-2.63
		Prevotellaceae unnamed genus	decrease	11	-2.29
		DA101	increase	11	1.74
		Clostridium	decrease	10	-2.36
		Desulfovibrio	decrease	10	-2.29
T2 non-responders	**Level**	**ASV**	**T2 vs. T1**	**W value**	**F statistic**
	Phylum	*Tenericutes*	decrease	7	2.98
	Class	*Mollicutes*	decrease	8	2.83
	Order	*Anaeroplasmatales*	decrease	9	-2.84

3.5 Discussion

We present here the results of the first observational, uncontrolled clinical trial involving feeding of an amino acid-based diet to manage canine CE. Key study findings were that the diet was palatable and that 16/23 dogs achieved clinical remission. Feeding of EL altered the fecal microbiome of dogs within the R group, suggesting a potential explanation for the lessened clinical disease activity. These findings indicate that feeding EL could be effective for the management of canine CE and should be evaluated in future controlled studies.

In our study, 22/23 dogs readily ate EL and remission from CE was achieved in 70% of dogs consuming EL. Clinical improvement was rapid, in concordance with previous studies observing positive responses within the first 10 to 14 days of dietary therapy for CE dogs[1, 3, 5, 22]. In studies involving hydrolyzed protein diets, 66 to 89% of CE dogs achieved clinical remission[2,3,5]. In the current study, 8/9 dogs previously failing a feeding trial with a hydrolyzed protein diet achieved clinical remission with EL. Other factors (lack of exclusive feeding, inadequate duration, concurrent comorbidities, unaddressed hypocobalaminemia) may have negatively impacted the success of historical diet trial; these factors could not be fully explored given the vagaries of owner memories and existing medical records. Our results reinforce the importance of providing clear guidance for proper implementation of a diet trial. Moreover, failure to improve on one diet does not rule out the possibility of diet-responsive CE in dogs[2,18,23].

Various mechanisms could explain the clinical improvement observed in the dogs in this study, including reduced antigenicity, altered nutrient profile, inclusion or exclusion of specific ingredients, and modification of the intestinal microbiome. Diet is a modifiable environmental risk factor for the onset and perpetuation of intestinal inflammation [24]. A direct beneficial effect of feeding free amino acids is possible. One study found reduced pro-inflammatory cytokine release when inflamed human intestinal biopsies were incubated with amino acids from an ED ex vivo [25]. Exclusion of dietary antigen could also reduce inflammatory stimuli. While food-derived antigens trigger immunological reactions in human Celiac disease (gliadin [26]) and IgE-mediated food allergy [27], there is minimal evidence that food proteins drive canine CE [28]. The ability of dietary protein to aggravate intestinal inflammation is also controversial in human IBD. Of note, one meta-analysis of CD patients treated with EEN found no difference in remission rates comparing utilization of diets sourcing protein from individual amino acids to those formulated with oligopeptides or intact proteins[29]. A head-to-head study comparing feeding of an

intact protein to an amino acid-based diet to CE dogs with immunological monitoring would be required to determine the importance of protein source in this disease.

Fat is another nutrient of interest in canine CE which can be a double-edged sword. Maldigestion of fat may provoke malabsorption and ensuing osmotic and/or secretory diarrhea[30,31]. Moreover, dietary fat has been linked to increased intestinal permeability and aggravated immune responses locally and systemically in mice[32]. Nonetheless, fat represents the most efficient means of delivering calories on a kilocalorie per kg diet basis. The fat in EL was provided via vegetable, coconut, and fish oils, resulting in an energy density of 3322 kCal/kg and a fat digestibility of 95.5%. Feeding of EL (23.7% fat on metabolizable energy basis) represented a reduction in dietary fat for 10/15 Rs and 5/5 NRs (original diet macronutrient data available for 20/22 dogs ingesting EL), so it does not appear that clinical response can be linked to fat restriction alone. Canine CE patients are nonetheless a heterogeneous group and so provision of a moderate amount of readily assimilable fat may have contributed to the clinical improvement seen in a subset of these dogs. Given the well-recognized benefit of diet modification in CE in dogs[1,5,6,12,33], there is a need to consider other factors connecting food components to GI-related clinical signs. A preponderance of data points to the ability of diet to modulate the GI immune system, with an indisputable role of the gut microbiome[32,34,35]. Recent studies indicate that the antigenic target in canine CE may be the intestinal microbiota rather than food components[11], raising the possibility that microbiome shifts may underlie clinical improvement. Feeding EL had significant effects on the fecal microbiome which varied based on T2 clinical response (Figures 3.4, 3.5). This finding is in line with studies in dogs[3] and humans[36] linking clinical response to diet with microbiome shifts. Contrary to results of studies employing polymeric diets for EEN in human IBD patients[36,37,38], changes did not include a decrease in bacterial richness or evenness with EL feeding.

How exactly changes in microbiome composition impacted T2 clinical outcome is unclear. It is possible that Rs benefited from the expansion of *Verrucomicrobia*. This phylum

includes mucus-degrading *Akkermansia* spp. whose abundance are inversely correlated to disease severity various chronic GI conditions[39]. Decreases in sulfate reducing *Desulfovibrionales* bacteria may have promoted clinical improvement by limiting luminal hydrogen sulfide, a compound known to promote intestinal inflammation[40]. Our approaches did not allow for assessment of total bacterial load, which has been found to be reduced with ED feeding[41]. We also acknowledge that analysis of the intestinal mucosal microbiome may have yielded more immunologically relevant information. However, serial biopsy samples are impractical, and data from humans has identified comparable alpha and beta diversity comparing intestinal mucosal and fecal samples[42]. Additional studies employing multiomics approaches are necessary to explore the functional consequences of these microbiome changes.

Unlike the fecal microbiome analysis, we found no utility of monitoring serum inflammatory biomarkers (cytokines, CRP) in these dogs. We attribute this to minimal systemic inflammation in our study cohort. This is not a novel conclusion, with many previous investigations finding CRP to be within the reference range in most or all normalbuminemic CE dogs[1,12,43]. CRP has also failed to correlate with CE disease activity44. Our findings encourage analysis of GI-relevant samples, such as feces and biopsy tissue. Serum B12 concentrations were adequate (>300 ng/L) in all dogs remaining in the study at T3; this includes 5 dogs that were hypocobalaminemic at study enrollment and 2 with historical hypocobalaminemia. Of note, urine MMA was not evaluated, so B12 deficiency at the cellular level cannot be ruled out[45]. The circulating half-life of B12 in healthy dogs is about 2 months[46,47], and so T3 concentrations likely reflect dietary uptake rather than exogenous supplementation. It is possible that EL feeding improved intestinal absorption in Rs.

Feeding of EL was not associated with owner-reported adequate relief from CE in 6/22 dogs. We elected to utilize the owner's response to the "adequate relief" question to determine clinical response given the inherent limitations of the CCECAI score and well recognized utility

of this parameter in human IBS studies[16]. At T2, 4/22 dogs met the R criteria due to CCECAI drop but their owner felt CE was uncontrolled. Two of 4 dogs exited the study between T2 and T3 and were ultimately classified as NRs. Thus, this question appears to be a useful adjunctive parameter beyond the CCECAI. Neither age, baseline CCECAI, serum albumin or B12 concentration, or presence of lacteal dilation were useful in predicting response to EL. The Rs had a median CCECAI of 6.5 (3-8) and age 2.75 (0.9-11) years and thus are in line with publications reporting a wide range of ages and disease severity in diet-responsive CE dogs[12,48,49]. Our results conflict with previous reports finding diet-responsive CE dogs to be younger, with lower clinical activity indices, versus dogs not responding to diet[1, 6]. Given that diet-responsive CE dogs tend to have better outcomes compared to other strategies[6], multiple dedicated feeding trials are prudent before resorting to immune suppressants or antibiotics.

Serum ALK-PHOS activity doubled in 11/22 dogs fed EL in the current trial, with values rising above the reference range in 5 dogs. One study in rats documented marked increases in hepatic lipid and cholesterol and significant reduction in fecal bile acid (BA) excretion during feeding of an ED[50]. In those animals, fatty liver was theorized to be secondary to reduced cholesterol catabolism for BA synthesis. A separate study in rats showed a significant increase in jejunal and ileal mucosal ALK-PHOS activity with EL feeding[51]. We did not measure ALK-PHOS isoenzymes or fecal BA concentrations, nor were hepatic biopsies obtained from any of the study dogs. Clearly, EL exerts strong metabolic pressures. Further study is required to determine the implications of these initial observations.

This study is not without limitations. It was uncontrolled, so we cannot draw any conclusions regarding the relative efficacy of EL compared to other diets and treatment modalities. Formal randomized trials are required to make these comparisons. Medications administered throughout the feeding period could have impacted clinical response, however, these were not changed during the study period, and so it is unlikely that clinical improvement

was falsely attributed to EL. Finally, fecal microbiome was only analyzed at T2; the impact of longer-term feeding is unknown.

3.6 Conclusions

Canine CE is a frustrating condition that may substantially reduce dog and owner quality of life. Diet-responsive dogs make up the largest subset of CE patients[1,22,49]. Due to lack of biomarkers able to predict diet-responsiveness, dedicated feeding trials are of utmost importance. Another option in the therapeutic armament may result in more dogs achieving long-lasting remission from their CE. Our results support a trial with EL in non-anorexic CE dogs before ruling out diet-responsive disease and further underscore the importance of the microbiome in chronic intestinal diseases.

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CHAPTER 4: TRANSCRIPTOMIC COMPARISON OF DUODENAL ENDOSCOPIC BIOPSIES FROM HEALTHY DOGS TO DOGS WITH CHRONIC ENTEROPATHY

4.1 Summary

Chronic enteropathy (CE) is a common but poorly understood condition in dogs. Intestinal inflammatory infiltrates are implicated in the pathogenesis, but evidence is lacking. We sought to utilize next-generation transcriptomic sequencing to compare gene expression in disease-relevant duodenal biopsies from healthy dogs to those from CE dogs. RNA was extracted from endoscopically obtained duodenal biopsies using a commercial kit and RNA sequencing was performed. Gene expression was compared with DESeq2 and pathway analysis was completed with gene set enrichment analysis. The study population consisted of 7 client-owned dogs with CE and a presenting complaint of diarrhea with disease severity ranging from mild to severe based on canine chronic enteropathy clinical activity index. Three male healthy beagle dogs comprised the control population. Principal components analysis separated 2 of 3 of the healthy dogs from the CE dogs. Differential expression analysis highlighted 1,138 genes with lower relative expression in CE compared to healthy and 68 genes with enhanced relative expression in the CE dogs. Genes with reduced expression in CE were related to extracellular matrix (ECM) maintenance and immunoglobulin-related genes. Upregulated genes pointed to contributions from epithelial cells. Enhanced anti-viral responses in CE were suggested by pathway analysis, as were disruptions of cell communications and ECM remodeling. These findings call into question current theories regarding an immune-driven cause of CE and support the power of transcriptomic analysis of intestinal biopsies to achieve enhanced resolution of the molecular changes present in the gut in CE.

4.2 Introduction

Many dogs suffer from persistent or relapsing gastrointestinal (GI) clinical signs such as diarrhea, poor appetite, and vomiting. Once specific causes for these signs have been ruled out, they are assigned the diagnosis of exclusion, chronic enteropathy (CE)[1]. Despite being the most common reason for chronic GI clinical signs[2,3], this condition is poorly understood. Classification systems beyond retrospective assignments based on response to therapy are utterly lacking. Thus, management involves a trial-and-error approach involving dietary, antibiotic, and/or immunosuppressant interventions and hoping for clinical improvement[4]. Most dogs improve clinically with diet, but between 15 to 40% may be non-responsive to all interventions[1].

Current theories regarding the pathogenesis of CE are largely extrapolated from GI conditions seen in other species. They revolve around an overzealous host immune response to harmless environmental (e.g., commensal bacterial, dietary) antigens[5,6]. However, data from canine patients with CE to back up these theories are insufficient. Traditional approaches, such as quantifying serum and mucosal cytokine concentrations[7,8,9,10,11], grading intestinal biopsy histopathological lesions[12,13,14], and cataloging intestinal bacterial populations[15,16] have been unable to disentangle the differences between healthy dogs and those with CE. A lack of dog-specific reagents, as well as lack of baseline knowledge from which to develop logical evidence-based hypotheses surrounding CE pathogenesis, impair progress on this front.

More comprehensive, less biased approaches are needed to bridge this gap. Bulk mRNA-sequencing (RNA-seq) offers a powerful means of gauging cellular activities in a high throughput manner[17,18]. The transcriptome provides a snapshot of gene expression, providing insight into what cells are doing rather that what cells are present. In CE, the primary site of disease appears to be the intestinal mucosa[19]. Histopathologic lesions within endoscopically obtained duodenal and colonic biopsies correlate the most strongly with clinical disease severity[20]. Most studies evaluating gene expression in intestinal biopsy samples from CE dogs have utilized real time RT-PCR to compare the expression of 1 or a handful of genes between

samples[7]. Results have been inconsistent, but a few groups have found elevated IL-12 expression in CE tissues from dogs with disease localized to the small intestine compared to healthy[21]. Analysis of micro-RNA expression within colonic mucosal biopsies from dogs with CE with disease localized to the large intestine highlighted differential expression in select markers identified in studies of various human diseases[22]. To our knowledge, there have been no RNA-seq studies duodenal biopsies in dogs with CE performed to date.

In this study, we used bulk RNA-sequencing to compare gene signatures in samples endoscopically obtained duodenal mucosal from healthy beagles to those from a client-owned dogs with CIE. We identified numerous differentially expressed genes and differentially represented pathways that provide a foundation to guide further study of the pathogenesis of CIE.

4.3 Materials and Methods

Study Population

The CE group consisted of client-owned dogs undergoing endoscopy as part of a clinical trial at Colorado State University's (CSU) Veterinary Teaching Hospital evaluating the efficacy of a novel diet sourcing protein from individual amino acids to manage clinical signs of CIE (Manchester 2023). This study was approved by CSU Institutional Animal Care and Use Committee (IACUC; protocol #1440). All dogs underwent a thorough workup to exclude metabolic, infectious, endocrine, and neoplastic causes for CE signs. This included a complete blood count, chemistry panel, urinalysis, abdominal ultrasound, fecal parasite screen, basal cortisol, and quantification of serum vitamin B12 and canine trypsin-like immunoreactivity (cTLI) levels. Eligibility requirements included uncontrolled CIE with current management, a canine clinical activity index (CCECAI) ≥3, no evidence of intestinal parasitism, and serum albumin >2 g/dL. Informed consent was obtained from all owners prior to endoscopy.

The healthy control (HC) group consisted of research colony beagle dogs. Duodenal samples were collected from the beagles independently from the diet trial, under a different CSU IACUC approved study (protocol #3002). The beagles were individually housed and maintained on the same diet with ad libitum water. Dogs were considered healthy based on absence of clinical signs and normal physical exam. They were screened with a complete blood count, chemistry panel, and quantification of serum vitamin B12 and cTLI levels.

Sample Collection and Processing

Both groups of dogs were fasted for 12 to 24 hours before being anesthetized in routine fashion for upper GI endoscopy. Upper GI endoscopy was completed, including evaluation of the esophagus, stomach, and the proximal duodenum. Biopsies were collected from the proximal duodenum, avoiding visible lymphoid aggregates, with 6 to 10 biopsies preserved in formalin for histopathologic evaluation, and another 4 biopsies preserved in RLT buffer for subsequent RNA extraction. Samples in formalin were submitted to the CSU Veterinary Diagnostic Laboratory for routine histopathologic evaluation and samples in RLT buffer were homogenized with a P1000 pipette before being frozen at -80°C until RNA extraction.

Biopsies in RLT buffer were thawed before RNA was extracted using Qiagen's RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the instructions provided by the manufacturer. RNA was quantified using a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA was stored at -80□C until analysis.

RNA-sequencing and Analysis Pipeline

Bulk RNA-sequencing was completed as previously described[23]. In brief, extracted RNA was sent to Novogene Corporation (San Diego, CA, USA) for Illumina-based sequencing. Libraries were generated using a NEBNext Ultra RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA) according to manufacturer's instructions. Paired-end reads, 150 bp in length,

were generated and used to create de-multiplexed fastq files. RNA-sequencing analysis was accomplished using Partek Flow software, version 10.0 (Partek Inc., Chesterfield, MO, USA). Raw reads were filtered for adapters and reads with N >10% and Phred scores >30. Reads were aligned to the CanFam3.1 genome assembly using STAR 2.7.3a[24]. Subsequent annotation and counting was completed with Ensembl 107 HT-seq[25].

Differential Gene Expression, Pathway Analysis and Statistical Comparisons

Differential gene expressed genes were identified with DESeq2[26]. Genes considering significantly differentially expressed between the HC and CIE samples included those with log(fold) change > 2 or < -2, with false discovery rate (FDR) adjusted *P* value of 0.05. Biological interpretations included gene ontology and gene set enrichment analysis (GSEA), (https://www.gsea-msigdb.org/gsea/index.jsp). Gene sets Hallmarks v2022.1, biocarta v2022.1, KEGG v2022.1, Gene Ontology go.bp v2022.1, and ImmuneSigDB v2022.1 were used for comparisons. Significant pathways were filtered using FDR *q* value of ≤ 0.25 and NOM *P* value ≤0.05. Individual pathway scores were also generated using GVSA (gene set variation analysis), canonical pathways, and Hallmarks[27]. (https://bioconductor.org/packages/devel/bioc/vignettes/GSVA/inst/doc/GSVA.html). The data included in this publication will be deposited in NCBI's Gene Expression Ombibus and are accessible upon contacting the corresponding author.

4.4 Results

Study Populations

The CE dog group consisted of 7 dogs, representing a range of ages and breeds (Table 4.1). All dogs had persistent small bowel diarrhea as a presenting complaint. Median CCECAI was 6.5 (range, 5-13). Abnormal laboratory findings at the time of inclusion were hypocholesterolemia in 1/7 dogs and hypocobalaminemia (<250 ng/L) in 4/7 dogs. Duodenal

biopsy histopathology results were compatible with CIE in all cases, with the most common finding being lymphoplasmacytic (LP) and eosinophilic enteritis in 5/7 dogs, graded as moderate in all 5. One dog moderate LP enteritis without notable eosinophils, and another had severe plasmacytic, eosinophilic, and neutrophilic enteritis (CE_3). Morphological lesions were mild to non-existent, apart from 2 dogs where mild lymphangiectasia was noted. Six of 7 dogs were initially prescribed a veterinary therapeutic diet, in keeping with the clinical trial. The seventh dog was diagnosed with perianal fistulae and was treated with cyclosporine (5.5 mg/kg PO q24h), which resulted in remission from CE signs and resolution of the perianal fistulae. Of the 6 dogs treated with diet, 3 were diet-responsive and 3 failed to improve with diet. Ultimately, 1/3 had complete resolution of signs on oral amoxicillin clavulanic acid, one improved on a home-cooked diet and budesonide, and the third dog continued to have non-responsive CIE (diarrhea, weight loss) 12 months after study participation.

The healthy beagles all had normal albumin, cholesterol, and vitamin B12 levels. Histopathologic analysis of the biopsies revealed mild LP enteritis in 1/3 dogs and moderate LP enteritis in 2/3 dogs, one of whom also had mild neutrophilic enteritis (HC_3). No morphological lesions were present in the biopsies from the beagles.

Table 4.1. Signalment and clinicopathological details of the 10 study dogs. Red values are below the reference range. CE, chronic enteropathy; H, healthy control; CCECAI, canine chronic enteropathy clinical activity index; alb, albumin; chol, cholesterol; GSD, German Shepherd dog.

ID	age (y)	sex	wt (kg)	breed	CCECAI	alb (g/dL)	chol (mg/dL)	B12 (ng/L)
H_1	6	M	14.4	Beagle	0	3.5	204	525
H_2	5	M	10.4	Beagle	0	4.3	245	492
H_3	6	M	13.4	Beagle	0	3.7	154	763
CE_1	5.5	MN	39	Great Pyrenees	8	3.3	152	227
CE_2	6.5	MN	31.3	Standard Poodle	6	3.5	243	352
CE_3	2	MN	25.7	Great Dane	13	3.0	82	150
CE_4	0.9	MN	22.8	Labrador retriever	5	3.5	186	179
CE_5	3.5	MN	28.8	GSD	12	3.0	151	1000
CE_6	11	MN	11	Boston terrier	3	3.5	197	234
CE_7	8	MN	36	GSD	9	3.1	250	338

Duodenal Biopsy Transcriptome Analysis Differentiates CE from Healthy

Our first question involved whether bulk RNA-seq of duodenal mucosal biopsy samples could differentiate HC from CIE dogs. In all, 13,602 protein coding genes were identified within the 10 samples. Principal components analysis (PCA) was used to visually evaluate transcriptomic similarity between the samples. Gene signatures accounted for 96.4% of the variability within samples, with 84.1% represented along the x-axis. Samples from 2/3 HC dogs clustered separately from the 7 CE dogs, with 1 HC dog (H_1) clustering closer to the CIE samples along the x-axis (Figure 4.1a). The CE dogs spanned the y-axis, and were also clustered along the z-axis, indicating separate subgroups within the CE populations (Figure 4.1b). Thus, gene expression in duodenal mucosal biopsies informs comparison of healthy and CIE.

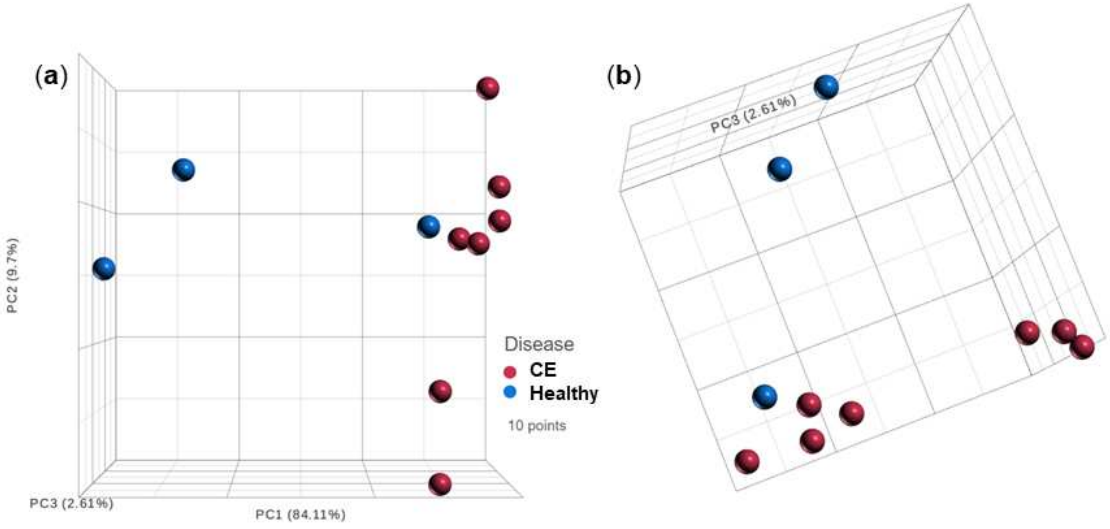
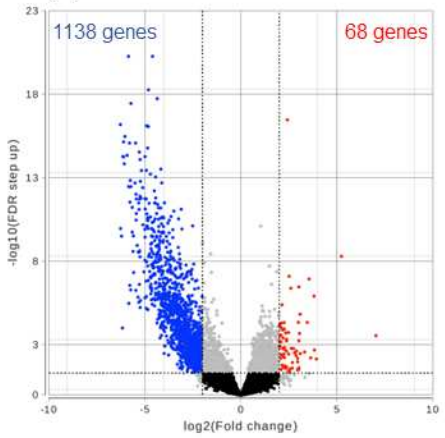


Figure 4.1. Principal component analysis (PCA) plot comparing transcriptomic analysis of duodenal biopsy RNA from healthy control beagles (n=3; blue dots) to client-owned dogs with chronic enteropathy (CE; n=7; red dots).

(a)



(b)

gene ID	gene description	FDR step up (CE vs HC)	Log2(Ratio) (CE vs HC)
UPREGULATED CE vs. HC			
CYP1A1	Cytochrome P450 1A1	2.94E-04	7.03
CLCA4	Chloride channel accessory 4	5.16E-09	5.24
PDZK1IP1	PDZK1 interacting protein 1	7.29E-03	3.93
PLA2G4F	Phospholipase A2 group IVF	2.13E-03	3.83
SLC5A8	Solute carrier family 5 member 8	1.26E-06	3.81
GPX2	Glutathione peroxidase 2	6.21E-03	3.64
ATP10B	ATPase phospholipid transporting 10B (putative)	1.18E-07	3.56
ENSCAFG00000042312	V-SNARE coiled-coil homology domain-containing protein	3.43E-03	3.10
ENSCAFG00000024408	UPAR/Ly6 domain-containing protein	1.51E-05	3.08
GSDME	Gasdermin E	2.16E-04	3.05
FAM83B	Family with sequence similarity 83 member B	5.87E-04	3.02
ANKRD1	Ankyrin repeat domain 1	2.47E-02	3.02
POF1B	POF1B actin binding protein	3.52E-07	3.02
PRSS54	Serine protease 54	3.19E-02	3.00
RNF39	RING finger protein 39	1.08E-02	2.96
SLC6A18	Inactive Na-dependent neutral amino acid transporter	3.28E-02	2.95
DOWNREGULATED CE VS. HC			
CCN1	Cellular communication network factor 1	6.60E-17	-6.27
ENSCAFG00000043925	Ig-like domain-containing protein	3.19E-10	-6.21
ENSCAFG00000046771	Ig-like domain-containing protein	1.02E-04	-6.17
ADAMTS1	ADAM metallopeptidase thrombospondin type 1 motif 1	5.68E-15	-6.11
COL15A1	Collagen type XV alpha 1 chain	1.49E-14	-6.07
CEMIP	Cell migration inducing hyaluronidase 1	3.41E-16	-6.04
ENSCAFG00000031273	Ig-like domain-containing protein	4.60E-15	-5.93
NIM1K	NIM1 serine_threonine protein kinase	3.46E-13	-5.84
ENSCAFG00000015176	Ig-like domain-containing protein	3.42E-06	-5.84
POU2AF1	POU class 2 homeobox associating factor 1	8.45E-16	-5.80
IGLV2-33	Immunoglobulin Lambda Variable 2-33	2.84E-07	-5.78
BHLHA15	Basic Helix-Loop-Helix Family Member A15	2.93E-12	-5.78
VWF	von willebrand's factor	1.47E-13	-5.75
FLT1	Fms related receptor tyrosine kinase 1	5.27E-07	-5.74
CNN1	calponin 1	3.57E-18	-5.72
ENSCAFG00000047763	IGv domain-containing protein	3.43E-13	-5.72
COL6A5	Collagen alpha-5(VI) chain	1.82E-10	-5.66
CD34	Hematopoietic progenitor cell antigen CD34	2.63E-09	-5.61
ENSCAFG00000049976	Ig-like domain-containing protein	3.07E-10	-5.60
ENSCAFG00000031403	Ig-like domain-containing protein	4.84E-08	-5.58

Figure 4.2. (**a**) Volcano plot depicting differentially expressed genes in duodenal biopsy samples between chronic enteropathy dogs (CE; n=7) and healthy control beagles (HC; n=3) based on DESeq2 analysis after selecting for genes with log(2)fold change >4 or < -4, with FDR *P* value < .05. Red dots represent genes significantly upregulated in CE vs. HC and blue dots represent genes downregulated in CI=E compared to HC. (**b**) Table displaying top DE genes in CIE compared to HC based on DESeq2.

With that established, we completed differential gene expression analysis was implemented to determine the drivers of the clustering seen on the PCA plot. After filtering for genes with fold change >2 or < -2 and FDR *P* value < 0.05, 1206 genes were identified as DE. Of these, 68 genes showed enhanced expression in CE samples compared to healthy, while 1,138 genes showed diminished expression in CE samples compared to healthy (Figure 4.2). Among the most upregulated genes from CE samples were CYP1A1, CLCA4, SLC5A8, and GPX2, suggesting an important role for intestinal epithelial cells. Conspicuously absent from the

tope DEGs overexpressed in CE samples were genes encoding cytokines, chemokines, and other pro-inflammatory mediators. Among the most downregulated genes in CE samples compared to healthy included CCN1 and ECM related genes ADAMTS1 and COL15A1. This finding could indicate an important role for stromal cells in CE pathogenesis. A group of stromal cells have been implicated in a subset of Crohn's disease patients refractory to anti-TNF drugs[28]. Additionally, 4 of the top 10 most DE genes which showed reduced relative expression in CE compared to health were unannotated genes with immunoglobulin-like functions. Reduced relative expression of these genes could point to differences in humoral immune responses in CE dog.

Pathway Analysis Implicates Anti-Viral Responses and Metabolic Dysregulation in CE

Integration of the gene sets identified as DE between health and disease may provide more biologically relevant information, so we performed gene set enrichment analysis. In general, normalized enrichment scores (NES) were slightly greater for pathways showing lesser relative expression in CE compared to health (Table 4.2, Figure 4.3). The most downregulated pathway in CE was epithelial to mesenchymal transition (EMT). Other pathways with lowest relative expression in CE dogs involved cell adhesion molecules and extracellular matrix interactions. Several pathways were significantly enriched in samples from CE dogs compared to health. Despite minimal inflammation-associated genes in the DE analysis, the top upregulated pathway was Hallmark interferon response. That, along with enhanced expression of the KEGG RIG-I like receptor and peroxisome pathways, suggests an anti-viral like response in the duodenal mucosa in CE[29], and further implicates a role for intestinal epithelial cells[30]. This could be a response to undetected viral infection in CE patients or off-target immune reactions against food antigens or disrupted gut microbial populations. Many of the other enriched pathways in CE compared to healthy involved metabolic pathways (Hallmark oxidative phosphorylation, fatty acid metabolism, KEGG propionate metabolism). Overall, pathway

analysis highlighted roles for cellular processes and tissue phenomenon not typically examined in relation to CE pathogenesis.

Table 4.2. Pathway analysis based on gene set enrichment analysis (GSEA). NES, normalized enrichment score; CE, chronic enteropathy; HC, healthy control.

pathway	gene count	NES, CE vs. HC	NOM p value	FDR q value
HALLMARK_INTERFERON_ALPHA_RESPONSE	73	2.449	0.000	0.000
HALLMARK_PROTEIN_SECRETION	88	2.397	0.000	0.000
BIOCARTA_MITOCHONDRIA_PATHWAY	18	2.153	0.000	0.004
BIOCARTA_DEATH_PATHWAY	26	2.125	0.000	0.003
HALLMARK_OXIDATIVE_PHOSPHORYLATION	156	2.099	0.000	0.000
KEGG_PROTEASOME	35	2.058	0.000	0.017
BIOCARTA_HIVNEF_PATHWAY	53	2.023	0.000	0.012
KEGG_RIG_I_LIKE_RECEPTOR_SIGNALING_PATHWAY	45	2.005	0.000	0.014
HALLMARK_FATTY_ACID_METABOLISM	125	1.977	0.000	0.001
HALLMARK_PI3K_AKT_MTOR_SIGNALING	93	1.967	0.000	0.001
KEGG_PEROXISOME	63	1.917	0.000	0.028
BIOCARTA_TOLL_PATHWAY	23	1.893	0.004	0.043
KEGG_PROPANOATE_METABOLISM	27	1.860	0.002	0.035
KEGG_CITRATE_CYCLE_TCA_CYCLE	26	1.809	0.002	0.046
HALLMARK_ANDROGEN_RESPONSE	88	1.697	0.000	0.008
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	155	-3.133	0.000	0.000
KEGG_COMPLEMENT_AND_COAGULATION_CASCADES	36	-2.471	0.000	0.000
HALLMARK_MYOGENESIS	140	-2.444	0.000	0.000
KEGG_ECM_RECEPTOR_INTERACTION	63	-2.414	0.000	0.000
KEGG_CELL_ADHESION_MOLECULES_CAMS	76	-2.246	0.000	0.000
HALLMARK_COAGULATION	104	-2.233	0.000	0.000
KEGG_GAG_BIOSYNTHESIS_CHONDROITIN_SULFATE	18	-2.226	0.000	0.000
BIOCARTA_EICOSANOID_PATHWAY	18	-2.166	0.000	0.001
KEGG_BASAL_CELL_CARCINOMA	40	-2.154	0.000	0.000
HALLMARK_KRAS_SIGNALING_UP	166	-2.096	0.000	0.000
HALLMARK_APICAL_JUNCTION	154	-2.053	0.000	0.000
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	157	-1.985	0.000	0.002
HALLMARK_HEDGEHOG_SIGNALING	29	-1.941	0.000	0.000
HALLMARK_UV_RESPONSE_DN	130	-1.862	0.000	0.001
KEGG_ARACHIDONIC_ACID_METABOLISM	30	-1.834	0.000	0.012

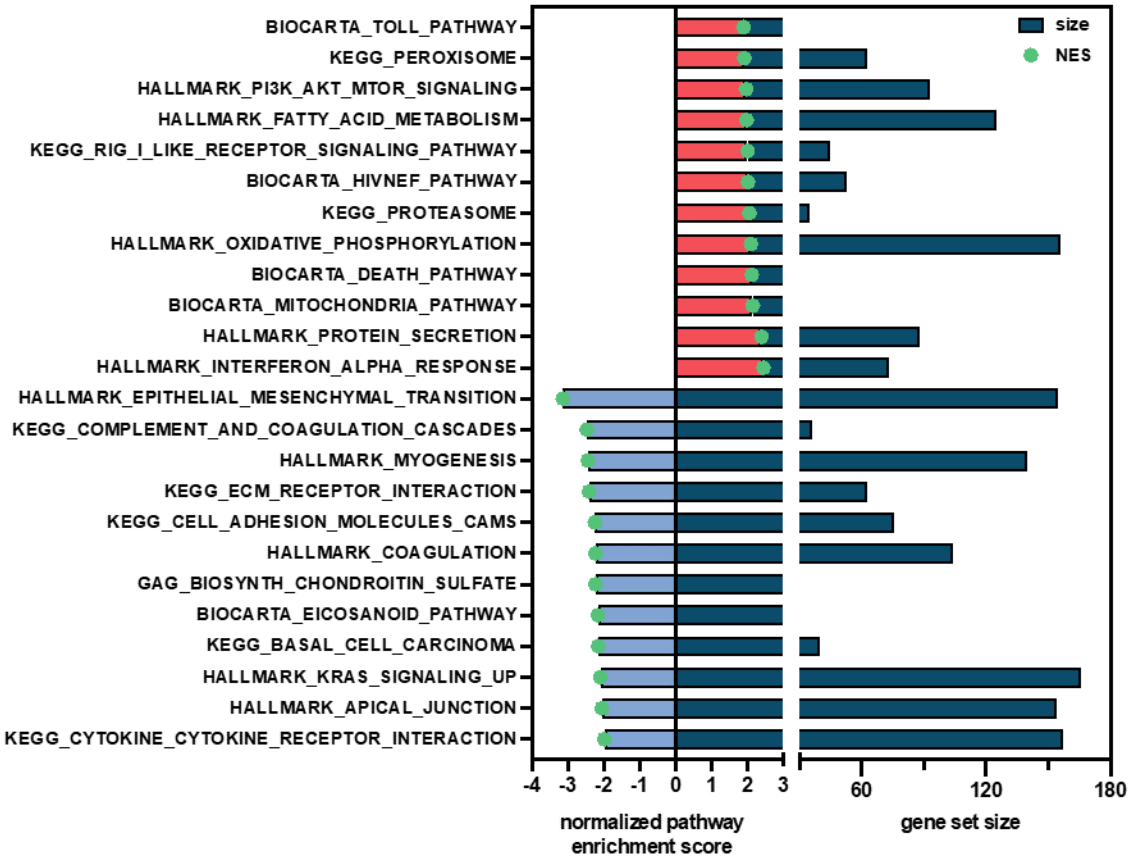


Figure 4.3. Bar chart showing results of gene set enrichment analysis (GSEA) using KEGG, Hallmark, and Biocarta pathways. Significant pathways organized by normalized enrichment score (NES); red bars are enriched in expression, CE compared to healthy and blue bars are reduced in expression. Navy bars indicate the number of genes within the pathway. All pathways displayed had FDR q value < .05.

4.5 Discussion

Chronic enteropathy is a heterogeneous and poorly understood syndrome manifesting as recurrent or persistent GI clinical signs. It is thought to be driven by intestinal inflammation and immune dysregulation, but this has not been definitively proven. Applying next-generation tools to compare intestinal mucosal tissues in health and disease is therefore a priority to improve understanding of the pathophysiology underlying this common condition.

In this study, we completed an initial comparison of bulk RNA-seq data from duodenal RNA from healthy beagles to that from client-owned dogs with CE. We have generated a comprehensive database of gene expression differences in disease-relevant tissues.

Comparison of gene expression in duodenal biopsies from CE dogs to those from healthy dogs identified hundreds of DE genes. Many more genes were downregulated than upregulated, in CE vs. health. This is of interest as disease-associated markers are typically enhanced in disease. Identifying markers based on their absence is more challenging but is a strength of bulk RNA-seq. With respect to pathway analysis, epithelial to mesenchymal transition was identified as the most downregulated pathway in CE samples. How this might impact CE pathogenesis is not exactly clear. This process would be expected to result in intestinal fibrosis, which has been correlated with disease severity in some histopathology-based studies of CE[20,31]. An inability of normal EMT to take place could result in disrupted intestinal barrier function and impaired mucosal healing[32]. Other downregulated pathways in CE samples further implicated tissue organization and cellular interactions. To better probe contributions of these processes to CE pathogenesis, we could complete spatial transcriptomics and single cell RNA-seq to interrogate intestinal barrier abnormalities in CIE more specifically.

Pathway analysis also yielded unexpected results, implicating anti-viral related responses in CE duodenum. Interferon, RIG-I, and peroxisome-related pathways are not among the immune mechanisms that are typically associated with CE in dogs[33]. These types of immune reactions have been implicated in Celiac disease, where single cell RNA-seq analysis identified an expansion of intra-epithelial lymphocytes with an anti-bacterial/anti-viral transcriptomic program in the duodenum of affected individuals[34]. Diet is the most effective strategy for clinical management of CE, enabling dogs to achieve remission without the need for medications[1]. This is like Celiac disease, where the mainstay of treatment is complete exclusion of gluten from the diet[35]. Until now, CE has been considered a canine version of other inflammatory bowel diseases of humans like Crohn's disease and ulcerative colitis. However, it is possible that CE

might be more analogous to Celiac based on our findings of the implicated immune pathways in this initial dataset.

There is relatively little existing data regarding gene expression in intestinal tissues from dogs with CE with which to compare our findings. The most comprehensive evaluation of duodenal gene expression was carried out in 2012 by Wilke et. al.[36]. This group analyzed RNA from duodenal biopsies from 18 CE dogs and 6 healthy mixed breed dogs using a 20,000 gene microarray. Their CE group of dogs included 5 with protein-losing enteropathy (PLE), which seemed to account for much of the differences they documented. In fact, they found just 1 gene, CXCL13, to be significantly differentially expressed between the dogs with severe CE but normal serum albumin and healthy dogs. These results provide further evidence that PLE is a distinct disease entity from CE[37], and points to the importance of well-defined disease phenotypes when studying CE. Our results did not implicate CXCL13 as a differentiator between CE and health, which may be due to distinct patient populations or the different methodological approaches.

Selection of the ideal healthy control population is problematic for studies of chronic enteropathies given a lack of objective markers of gut health. For this initial survey, we elected to utilize samples from healthy adult beagles showing no outward signs of GI dysfunction (e.g., normal fecal scores, good appetites, normal body condition and muscle condition scores). Nonetheless, one HC dog clustered closer to the CE dogs than the other HC dogs on the PCA plot; this calls into question whether the dog was truly healthy or if it had subclinical GI disease. For the purposes of this study, we did not exclude this dog from the HC group, but in an ideal world we would have data from a larger population of outwardly healthy dogs which we could screen for inclusion. Our healthy and CE populations were distinct in terms of breed and size, and so it is possible that some of the differences detected by our analysis are breed related. Earlier work suggests that beagles may have higher than normal small intestinal bacterial load[38,39], but this has not been established with modern analytical approaches. A future study

could utilize tissues from age- and breed-matched dogs to reduce some of the variability between the HC and CE groups. Finally, CE is a diagnosis applied to a heterogeneous group of patients. Larger sample sizes, including dogs with different clinical manifestations and disease severities, will be needed to accomplish our goal of comprehensively characterizing the molecular mechanisms at play in these patients.

4.6 Conclusions

We completed an RNA-seq analysis of duodenal biopsies from healthy dogs to those with CE. Analysis highlighted the presence of many DE genes, with a far greater number showing diminished expression in the duodenum of dogs with CE compared to health. Pathway analysis suggested that anti-viral responses may be overly active in CE, and that tissue organization and restitution may be abnormally downregulated. This approach underscores the comprehensive potential of RNA-seq analysis. With this data in hand, we can design future experiments based on these findings to explore CE pathogenesis in a logical, evidence-based, and minimally biased manner.

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Chapter 5: SINGLE-CELL RNA SEQUENCING INVESTIGATION OF THE DUODENAL MUCOSA IN CANINE CHRONIC INFLAMMATORY ENTEROPATHY AND HEALTH[3]

5.1 Summary

Chronic inflammatory enteropathy (CIE) is a common condition in dogs causing recurrent or persistent gastrointestinal clinical signs. Pathogenesis is thought to involve intestinal mucosal inflammatory infiltrates, but histopathological evaluation of intestinal biopsies from dogs with CIE does not guide treatment decisions, inform prognosis, or correlate with clinical remission. We employed single-cell RNA sequencing to catalog and compare the diversity of cells present in duodenal mucosal endoscopic biopsies from 3 healthy dogs and 4 dogs with CIE. Through characterization of 35,668 cells we identified 30 transcriptomically distinct cell populations, including T cells, epithelial cells, myeloid cells, and plasma cells. Both healthy and CIE samples contributed to each cell population. T cells were broadly subdivided into tissue resident and non-resident categories, with no quantitative differences among T cell subtype proportions comparing CIE to healthy. Among the myeloid cells, neutrophils from CIE samples exhibited inflammatory (SOD2 and IL1β) gene expression signatures. Numerous differentially expressed genes were identified in epithelial cells, with the most significantly differentially expressed gene encoding for a 2-pore potassium channel (KCNK16). Overall, this work reveals the previously unappreciated cellular heterogeneity in canine duodenal mucosa and provides new insights into molecular mechanisms which may contribute to intestinal dysfunction in CIE. The cell type gene signatures developed through this study may also be used to better understand the subtleties of canine intestinal physiology in health and disease.

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3. This chapter includes a version of a manuscript submitted for consideration for publication in Frontiers Immunology, section Comparative Immunology entitled “Single-Cell RNA Sequencing Implicates Neutrophil and Epithelial Contributions to the Pathogenesis of Chronic Inflammatory Enteropathy in Dogs” (manuscript ID 1397590).

5.2 Introduction

Chronic inflammatory enteropathy (CIE) in dogs is a syndrome characterized by clinical signs indicating gastrointestinal (GI) tract dysfunction lasting longer than 3 weeks. Diagnosis is achieved by ruling out organic causes for these signs including infectious, endocrine, metabolic, and neoplastic causes[1]. It is the most common reason for chronic diarrhea, vomiting, and/or poor appetite in dogs[2,3], and is managed with various strategies including diet, microbiota modulators, and immunosuppressants[4]. As such, canine CIE shares features with human irritable bowel syndrome (IBS), celiac disease, and inflammatory bowel diseases[5,6,7]. Dogs and humans share dietary aspects and living environments which may contribute to disease. Spontaneously occurring CIE is a relevant model for enteropathies in humans, which have been increasing in prevalence[8]. Advancements in understanding the pathogenesis of canine CIE are needed to improve outcomes, develop new therapies for dogs, and to advance the use of this condition as a translational model.

Histopathologic evaluation of intestinal biopsy samples is a common clinical tool used in the diagnostic workup; it has been employed to investigate CIE pathogenesis[9]. Biopsies are characterized based on the severity of mucosal inflammatory cell infiltrates and morphological lesions of the epithelium and lamina propria[10]. Updated grading schemes have attempted to reduce interobserver variability and subjectivity[11], but histopathologic evaluation of intestinal biopsies remains a tool of limited utility given overlap in histopathology findings between health and CIE[12,13] and absence of specific lesions to guide clinical management[14,15]. Immunohistochemistry (IHC) has improved the granularity of histopathologic evaluations, enabling quantification of T cell subsets, including Foxp3-expressing regulatory T cells[12,16]. Despite these advances, the utility of IHC is restricted by pre-existing knowledge of cell markers of interest, and inability to look at numerous markers simultaneously. More sophisticated

approaches are necessary to identify and characterize the diverse cell types present in the canine small intestinal mucosa.

Single-cell RNA sequencing (scRNA-seq) is a high-resolution molecular technique that enables the characterization of transcriptomic activity on an individual cell basis[17,18]. This approach overcomes traditional barriers to disease investigation (e.g., species-specific reagents) and enables characterization of rare cell subtypes among heterogeneous samples. Recently, two canine scRNA-seq reference datasets of peripheral blood mononuclear cells (PBMCs) and circulating αβ T cells have been generated, revealing numerous transcriptomically unique cell populations[19,20]. Application of scRNA-seq to celiac[21] and Crohn's disease[22] have provided new molecular insights, and substantially advanced our understanding of these complex conditions.

Given the translational relevance of canine CIE, we applied scRNA-seq to investigate the heterogeneity in canine duodenal mucosa to characterize cellular and molecular aspects of the disease. Through use of unsupervised clustering, cells were grouped according to transcriptomic similarity, and identities were assigned based on gene signatures. We identified 30 transcriptomically distinct cell subtypes, including intestinal epithelial, T cell, myeloid, and B cell populations. Our analysis revealed minimal CIE associated differences within T cell subsets, with more pronounced impacts on myeloid and intestinal epithelial cell gene expression profiles. Overall, our analysis provides valuable canine specific cell type gene signatures and provides important new insights into the pathobiology of CIE, from which further study can be completed.

5.3 Materials & Methods

Study Population

Dogs with CIE were selected during screening for a prospective clinical trial at Colorado State University Veterinary Teaching Hospital (CSU-VTH) involving feeding of novel diet

sourcing protein from individual amino acids (Colorado State University IACUC protocol #1440)[23].

Dogs were considered for inclusion in the CIE group if they (1) exhibited clinical signs compatible with chronic enteropathy for >3 weeks, (2) were not experiencing adequate relief from their CIE signs according to their owner, and (3) had a canine chronic enteropathy activity index (CCECAI) score ≥ 3. Extra-intestinal reasons for clinical signs were ruled out with a physical exam, complete blood count, chemistry panel, and assessment of serum cobalamin (B12), folate, canine trypsin like immunoreactivity (cTLI) and canine pancreatic lipase immunoreactivity (cPLI) concentrations. Exclusion criteria included serum albumin < 2.5 g/dL, treatment with antibiotics within 1 month of study enrollment, or immunosuppressive drugs within 7 days of GI biopsy collection.

Healthy dogs were selected from a colony of research beagles and were considered eligible for inclusion based on absence of clinical signs associated with CIE. The healthy beagles were screened with a physical exam, chemistry panel, and assessment of serum B12, cTLI and cPLI concentrations. Fecal samples from dogs had been screened with fecal flotation to identify intestinal helminth shedding within 2 months of sample collection.

Sample Acquisition

Dogs were fasted for up to 24 hours prior to endoscopy and anesthetized using routing protocols. The upper GI tract was inspected visually, and samples were collected for histopathology. In addition, 12 to 15 biopsies were collected from the proximal duodenum with single use biopsy forceps then placed into ice cold Roswell Park Memorial Institute (RPMI) 1640 medium with L-glutamine (Corning, Glandale, AZ) until processing.

Sample Processing

Biopsy samples were rinsed with ice cold phosphate buffered saline (PBS), then centrifuged at 400 rcf for 5 minutes. To dissociate epithelial cells the samples were digested in Hanks' balanced salt solution (HBSS; Gibco, ThermoFisher Scientific, Waltham, MA, USA) with 2 mM ethylenediaminetetraacetic acid (EDTA; ThermoFisher Scientific) and 10% fetal bovine serum (FBS; Peak Serum, CO, USA) for 30 minutes at 37°C with intermittent vortexing. Following the EDTA digestion, the supernatant was removed, passed through a 70 mM cell strainer (Greiner Bio-One, Monroe, NC), and stored in HBSS with 5% FBS and 10 mM 4-(2-Hydroxyethyl)-1-piperazineëthanesulfonic acid (HEPES) on ice. The remaining tissue was resuspended in HBSS with collagenase type II (Gibco, Grand Island, NY) at 250 U/mL and incubated for 30 minutes at 37°C with intermittent vortexing. The two cell fractions were pooled together then passed through a 40 mM cell strainer (Greiner Bio-One, Monroe, NC). The pooled cells were resuspended in 4 mls HBSS and layered on top of 3 mls of Ficoll-Paque PLUS (Cytiva, Uppsala, Sweden). Cells were isolated using density gradient centrifugation to enrich live cells for 30 minutes at 400 rcf with maximum acceleration and no brake. The cellular interface was aspirated then washed with PBS. If cell yield was low, all cells were pooled to obtain adequate cell numbers. To remove contaminating red blood cells, cells were incubated in ammonium-chloride-potassium lysis buffer at room temperature for 3 to 5 minutes. A final 15-minute centrifugation at 100 rcf at 8°C was performed to remove small cellular debris and platelets. Lastly, cells were resuspended in 0.04% molecular grade bovine serum albumin (Sigma-Aldrich; St. Louis, MO) in PBS and transported to a Chromium iX instrument (10x Genomics; Pleasanton, CA) for cell capture. All samples were captured within 30 minutes of dissociation.

Library Preparation and Sequencing

Single cells were isolated and tagged with molecular barcodes using a Chromium iX instrument with a target of 5,000 cells per sample (10x Genomics). Cellular cDNA was pooled and prepared for Illumina sequencing using Chromium Next GEM Single Cell 3' Kit v3.1 and dual index library construction kits (10x Genomics). Library quality was analyzed using a LabChip (PerkinElmer; Waltham, MA) and sequenced on an Illumina NovaSeq 6000 sequencer (Novogene Corporation; Sacramento, CA) with a target of 50,000 150 bp paired-end reads per cell. Raw data were demultiplexed by the sequencing core then transferred for downstream analysis.

Read Mapping and Quantification

A Cell Ranger analysis pipeline (version 6.1.2, 10x Genomics) was utilized to process raw FASTQ sequencing data, align reads to the canine genome, and generate a count matrix. The default settings were used when running “cellranger count” and aligned to a CanFam3.1 reference prepared as previously described[24].

Data Filtering, Integration, Dimension Reduction, and Unsupervised Clustering

The count matrix for each sample was imported into R using the Read10X() function then converted to a Seurat object using the CreateSeuratObject() function[25]. To estimate the number of dead/poor quality cells, the percentage of mitochondrial reads per cell was calculated using PercentageFeatureSet() to count all reads mapped to features with the prefix "MT-". Consistent with previous reports of scRNA-seq in human duodenal cells, we observed epithelial populations to have greater than 60% mitochondrial reads[26,27]. To account for the difference in mitochondrial feature percentages within immune and duodenal populations we completed two filtering steps to prepare the dataset for analysis. First, each object was filtered leniently to allow for retention of all cells except high end outliers using the following parameters: UMI counts per

cell (100 < nCount_RNA < 25000), unique feature counts per cell (200 < nFeature_RNA < 3000), and percentage of mitochondrial reads per cell (percent.mt < 80). Then, DoubletFinder was used to identify and remove putative cell doublets[28]. Following doublet removal, a second filtering step was applied to non-epithelial cells in which cells with greater than 12.5% of reads mapping to mitochondrial features were excluded from downstream analysis. After completing QC filtering on each sample, all samples were integrated into one object using a SCTransform() normalization and canonical correlation analysis (CCA) integration workflow using 2500 features as integration anchors[25]. During this step, we used the percent mitochondrial reads as a latent variable in a linear regression framework to minimize the impact of mitochondrial reads on dimension reduction and integration. Following data integration, one additional low-quality cluster (defined by low UMI counts) was identified and removed. Ideal clustering parameters for the complete dataset (res = 1.3 dims = 40, n.neighbors = 50, min.dist = 0.3) and the parameters for each cell type subset were determined using the R package clustree[29]. Dimension reduction and visualization was completed, and the data were projected using 2-dimensional, non-linear uniform manifold approximation and projection (UMAP) plots.

Cell Classification

Following unsupervised clustering, the FindAllMarkers() function in the Seurat package was used to identify the enriched features in each cluster relative to all others (test.use = “wilcox”, only.pos = TRUE). The gene lists were manually evaluated to identify enrichment of canonical markers as defined in canine and human cell types. Key features and references supporting the use of specific markers to define a cell type are presented in Supplemental table 5.1. To supplement manual annotation, we also used SingleR[30] and Seurat’s reference mapping protocol to transfer cell type annotations from a canine leukocyte atlas and the human gut cell atlas[24,31]. Following manual and algorithmic cell type identification, the cell type divisions derived from unsupervised clustering were then collapsed into biologically relevant cellular subtypes.

Both the collapsed clustering (primary figures) and results of unsupervised clustering (supplemental) are presented. This process was completed on the full integrated dataset, as well as each of the subsets analyzed through subset analysis, and the approach used clustree output at multiple clustering resolutions to inform the identification of parent clusters.

Feature Visualization

Gene expression was visualized using (1) colorization of expression (log transformed data) values on UMAP plots, (2) dot plots of expression (scaled, log transformed data) by cluster, or (3) violin plots of expression (log transformed data) by cluster. Expression of features between conditions (CIE versus healthy) were visualized by splitting the UMAP by condition then colorizing the plots by expression values of log transformed data. When completing visualization with a split UMAP plot, the conditions were down sampled to obtain equal cell numbers depicted in the UMAP plot for each condition.

Cell Abundance Analysis

Cell type and subtype percentages were determined for each sample with the denominator being the total number of cells present in the subset for a given sample. The cell type percentages were then used to complete a Wilcoxon rank-sum test to evaluate statistical significance between samples from CIE and healthy tissues. In addition to statistical approaches, the data were visually inspected in the UMAP space though use of colorization by sample as well as plotting using stacked bar graphs. For stacked bar graphs, the depicted percentages were calculated using the percentage of cells from a given sample out of all cells within a given cluster. To avoid bias from differences in cell numbers, all biological replicates were down sampled to obtain equal representation prior to the calculation of percentages.

Differential Gene Expression Analysis

Gene expression changes between cells obtained from CIE and healthy dogs were evaluated using both Wilcoxon rank-sum tests and pseudobulk conversion followed by a DEseq2 workflow[32]. Two approaches were utilized throughout because Wilcoxon rank-sum tests are prone to false positives, while pseudobulk differential gene expression (DGE) analysis is susceptible to masking biologically relevant changes due to an inability to perform well with zero-inflated datasets[33]. We first applied Wilcoxon rank-sum tests when completing DGE analysis and used threshold for adjusted P value of < 0.01 and a log2(fold change) > 0.58 to be considered statistically significant. Results of Wilcoxon rank-sum analyses were depicted as scatter plots of the average expression in CIE and healthy, with colorization by statistical significance red (adjusted P value of < 0.01 and a log2(fold change) > 0.58) and blue (adjusted P value of < 0.01 and a log2(fold change) < -0.58). After completing DGE analysis using Wilcoxon rank-sum tests, we then completed the same comparison using pseudobulk conversion. This approach consisted of removing features that had less than 10 cells with a non-zero expression value then collapsing raw counts data for each sample into one column. From there, sample by gene count matrices were analyzed using a DEseq2 workflow and statistical significance was evaluated based on an adjusted P value < 0.05 and a log2(fold change) greater than 0.58.

Gene Set Enrichment Analysis

Statistically significant differentially expressed features (between conditions or clusters) were used as input to complete gene set enrichment analysis using the enrichr() function from the clusterProfiler R package[34]. The “C5”, “C2”, and/or “Reactome” databases from the msigdbr package were used to complete enrichment analysis[35]. Significance was evaluated based on terms that achieved a Benjamini-Hochberg corrected P value < 0.05. Enriched terms were plotted using bar charts with the signed log10(P value) presented to depict significance.

Flow Cytometric Analysis

Remnant cells from the samples used for scRNA-seq were analyzed using flow cytometry to interrogate immune cell protein expression. Approximately 500,000 cells were used to complete immunostaining. Each sample of dissociated duodenal cells were stained with a panel of 4 antibodies (Supplemental table 5.2). Cells were washed with FACs buffer (5% FBS with 0.1% sodium azide in PBS), then blocked with 5 uL normal dog serum while incubated with a combination of the primary antibodies diluted in FACS buffer for 25 minutes at 4°C. Cells were washed with FACS before 5 uL 7-AAD (Invitrogen, Waltham, MA, USA) was added, and samples were run on a Beckman Coulter Gallios 3-laser flow cytometer. Flow data were analyzed with FlowJo software version 10.8.1.

Data and Software Availability

Raw sequencing data and cell by gene count matrices are available on the NCBI Gene Expression Omnibus database (GSE254005). The annotated dataset is available for browsing at the UCSC Cell Browser (url to be provided at time of publication)[36]. Processed data (Seurat objects), analysis code, and software versions are available at https://github.com/dyammons/canine_duodenal_atlas. Any additional data requests can be made by contacting the corresponding author.

5.4 Results

Patient Populations

The healthy dog cohort consisted of 3 adult colony intact male beagles. Physical exam and diagnostic test results (chemistry panel, fecal parasite screening) were unremarkable, and all dogs had normal serum cobalamin concentrations (Table 5.1). The CIE dogs included dogs of distinct breeds and inadvertently were all neutered males. Clinically, all presented with

chronic diarrhea (Table 5.1) and canine chronic enteropathy clinical activity indices (CCECAI) compatible with mild to severe disease[15]. Physical exam findings in the CIE dogs included low body condition score in 2 dogs with normal body condition scores in the other 2. Two dogs were hypocobalaminemic (serum vitamin B12 <250 ng/L).

Table 5.1. Study dog characteristics. CCECAI, canine chronic enteropathy clinical activity index. BCS, body condition score (based on scale of 1 to 9).

	ID	age (y)	sex	breed	weight (kg)	BCS	CCECAI	albumin (g/dL)	B12 (ng/L)	medications
Healthy	H_1	6	M	Beagle	14.4	5	0	3.5	525	none
	H_2	5	M	Beagle	10.4	5	0	4.3	492	none
	H_3	6	M	Beagle	13.4	5	0	3.7	763	none
CIE	CIE_1	5.5	MN	Great Pyrenees	39	3	7	3.3	227	none
	CIE_2	6.5	MN	Standard poodle	31.3	5	3	3.5	352	phenobarbital 3.1 mg/kg PO q12h, alprazolam 3.2 mg/kg PO q24h, gabapentin 9.6 mg/kg PO q12h, Visbiome Vet
	CIE_3	2	MN	Great Dane	25.7	1	13	3.0	145	mirtazapine 1.1 mg/kg PO q12h, Visbiome Vet, Fortiflora
	CIE_4	2.5	MN	Labrador retriever	29.1	5	9	3.1	>1000	cyanocobalamin 1000 mcg PO q24h

Duodenal biopsies from all study dogs were evaluated with routine histopathology by a single board-certified veterinary pathologist, and results are provided in Supplemental table 5.3. Samples from healthy dogs revealed evidence of mild (n=1) to moderate (n=2) lymphoplasmacytic (LPL) infiltrates with no morphologic lesions detected. One healthy dog had a mild neutrophilic infiltrate. These dogs were retained as healthy controls as the dogs lacked clinical signs of GI disease and LPL duodenal infiltrates are not specific for clinical disease[13,37,38,39]. Histopathologic conclusions in the CIE dogs included LPL enteritis with mild (n=3) to marked (n=1) neutrophilic infiltrates. A marked eosinophilic component, along with mild crypt dilation and epithelial surface injury, was also appreciated in one dog (CIE_3).

Generation of a Cellular Atlas From Canine Duodenal Mucosa

The first objective of this study was to generate an atlas of cells present in the adult canine duodenal mucosa. To accomplish this goal, we profiled the transcriptomes of 35,668 cells from duodenal biopsies obtained from 3 healthy and 4 CIE dogs. The average number of cells collected from each study dog was 5,095, and each sample was sequenced to an average depth of 43,956 reads per cell (Supplemental table 5.4).

Analysis of the integrated dataset revealed the presence of 7 major cell types which included epithelial cells, T cells, myeloid cells, mast cells, B cells, plasma cells, and a population of cycling T cells (Figure 5.1a, Supplemental data 5.1). Through subset analysis of the major cell populations, we were able to further divide the dataset into 30 transcriptomically distinct populations (Table 5.2, Supplemental figure 5.1a, Supplemental data 5.2). Annotation of the major cell types was completed using canonical markers, with reference mapping to human scRNA-seq duodenal data and a canine scRNA-seq leukocyte dataset (Figure 5.1 b/c, Supplemental table 5.1, Supplemental figure 5.1b-d)[24,31]. Briefly, epithelial cells were characterized by expression of sucrase isomaltase (SI), fatty acid binding protein 1 (FABP1), retinol binding protein 2 (RBP2), and apolipoprotein A1 (APOA1)[31,40,27]. T cells were identified based on expression of CD3E, CCL4, PTPRC (CD45) and IL7R[41,42]. Myeloid cells included cells exhibiting overexpression of allograft inflammatory factor 1 (AIF1), lysozyme (LYZ) and complement protein genes (C1QA, C1QB, C1QC)[31,43]. Plasma cells were enriched in expression of JCHAIN and retinoic acid receptor responder 2 (RARRES2) expression[19,40], while B cells were defined by MS4A1 (CD20), CD19, and PAX5 expression[19,31,43]. Mast cells exhibited relative overexpression of KIT, IGF1 and CD52[21,43]. Lastly, the population of cycling T cells was defined by expression of T cell markers with additional expression of TUBA1B, TOP2A and STMN1[40,43]. To assess the accuracy of major cell type annotation, we evaluated the correlation of relative cell type proportions as determined by scRNA-seq compared to flow cytometry (Supplemental figure 5.1e). The analysis revealed a strong correlation ($R^2 = 0.95$) with a slope of 0.97, indicating concordance of cell type classifications between the two approaches.

Table 5.2. Top upregulated genes comprising transcriptional signatures of canine duodenal cells.

Cell type	Major markers
T cells	
CD8_eff	GZMA, GZMB, CCL5, CD7, CD2, ITGB7, CD96, CD3E
Tnaive	VIM, IL7R, S100A8, EEF1A1, CXCR4, LTB, TMSB10
CD8_TRM	CCL4, RGS1, IFNG, NR4A2, BCL2A1, FASLG
gdT_1	GZMA, TRAT1, GZMB, TNFRSF6B, PTPN22, CCL5
NK_T	CRTAM, CTSW, CD160, TCF7, TESC, NME1, REL
CD8_mem	GZMK, FOS, SH2D1A, CCL4, CXCR4, KLF4, DLA-DRA
gdT_2	CCL5, SPNS3, GZMA, CAPG, IL2RB, TRAT1, CD7
Treg	TNFRSF4, TNFRSF18, S100A5, CTLA4, ICOS, NFKBIA
NK	KRLB1, NFKBID, NCR3, GADD45B, PRDX6, NFKBIA
T_IFN	ISG15, MX2, IFGGB2, OAS1, IFI44, SAMD9L, IFI44L
ILC2	MMP9, IL17RB, GATA3, PLAC8A, CLINT1, MPP4, ARL4C
Epithelial cells	
Enterocyte_1	ND4L, UGT1A6, SI, MYO1A, FAM13A, FAM15A, FAM151A
Enterocyte_2	APOC3, APOA4, APOA1, APOB, TM4SF20, CLCA4, SLC40A1
Enterocyte_3	FABP1, ATP5MC1, MGST3, COX4I1, RBP2, GSTP1, SLC25A5
BEST4+_enterocyte	GUCA2A, GUCA2B, CFTR, SYNC, STOM, CA7, BEST4
Goblet cell	ZG16, SPINK4, CLCA1, BCAS1, LYPD8, PNLIP, SYTL2
Tuft cell	ANXA4, CA2, TRPM5, IRAG2, RYR1, FYB1, SYNJ1, ATP2A3
IFN_enterocyte	ISG15, RSAD2, IFIT1, DDX60, APOC3, RNF213, OAS1
Stromal cell	TPM2, VIM, ADAMDEC1, IGFBP7, COL1A2, MYL9
Enteroendocrine cell	CHGB, ADGRG4, SCG2, AFP, UNC13B, PCSK1N, TPH1
Myeloid cells	
Neutrophil	S100A12, S100A8, SERPINA1, SOD2, NFKBIA, SELL, ISG20
Eosinophil	MMP1, MS4A2, CAT, PGF, CHI3L1, ADAMDEC1, FABP3
Monocyte	MT1E, MT2A, HMOX1, C1QC, C1QA, GSTP1, CTSS
Macrophage	CCL3, MAFB, C1QA, C1QC, STAB1, CTSS, CCL7, C1QB
cDC1	TMSB10, BATF3, ECRG4, CLEC1B, NAPSA, DNASE1L3
IL22RA _DC	TMBS10, CD52, ECRG4, MAL, S100A5, FBP1, CA2, LSP1
Plasma cells	JCHAIN, IGHM, TXNDC5, RARRES2, PRDX4, DERL3
Cycling T cells	TUBA1B, TOP2A, STMN1, CENPF, HMGB2
Mast cells	KIT, IGF1, CD52, CXCR4, SVIL
B cells	TNFRSF13C, DLA-DRA, FCRLA, SAMSN1, CD22, PAX5

Evaluation of cell type abundances revealed marked variability within both the healthy and CIE dogs (Figure 5.1d, Supplemental figure 5.2a). Statistical evaluation of major cell type proportions revealed no significant differences between conditions (Supplemental figure 5.2b).

Given this finding, to evaluate differential gene expression (DGE) analysis between all cells from CIE and healthy dogs (Figure 5.1e, Supplemental data 5.3). This analysis revealed few differentially expressed genes (DEGs), of which 70 features were enriched, and 55 features were downregulated in dogs with CIE. To further explore the origin of CIE-associated differences, we completed DGE analysis within each major cell subset. This analysis identified the greatest number of DEGs in myeloid, epithelial, and mast cells, while the fewest number of DEGs were found within B cell and T cell populations (Figure 5.1f, Supplemental data 5.3). The limited number of mast cells isolated precluded further investigation through subset analysis, so we completed gene set enrichment analysis (GSEA). The analysis revealed terms associated with aerobic respiration and interferon gamma responses to be enriched in mast cells obtained from CIE affected dogs, suggesting a potential contribution to the disease process (Supplemental figure 5.2c). From here, we investigated each major cell subpopulation individually to complete a more refined evaluation of the dataset.

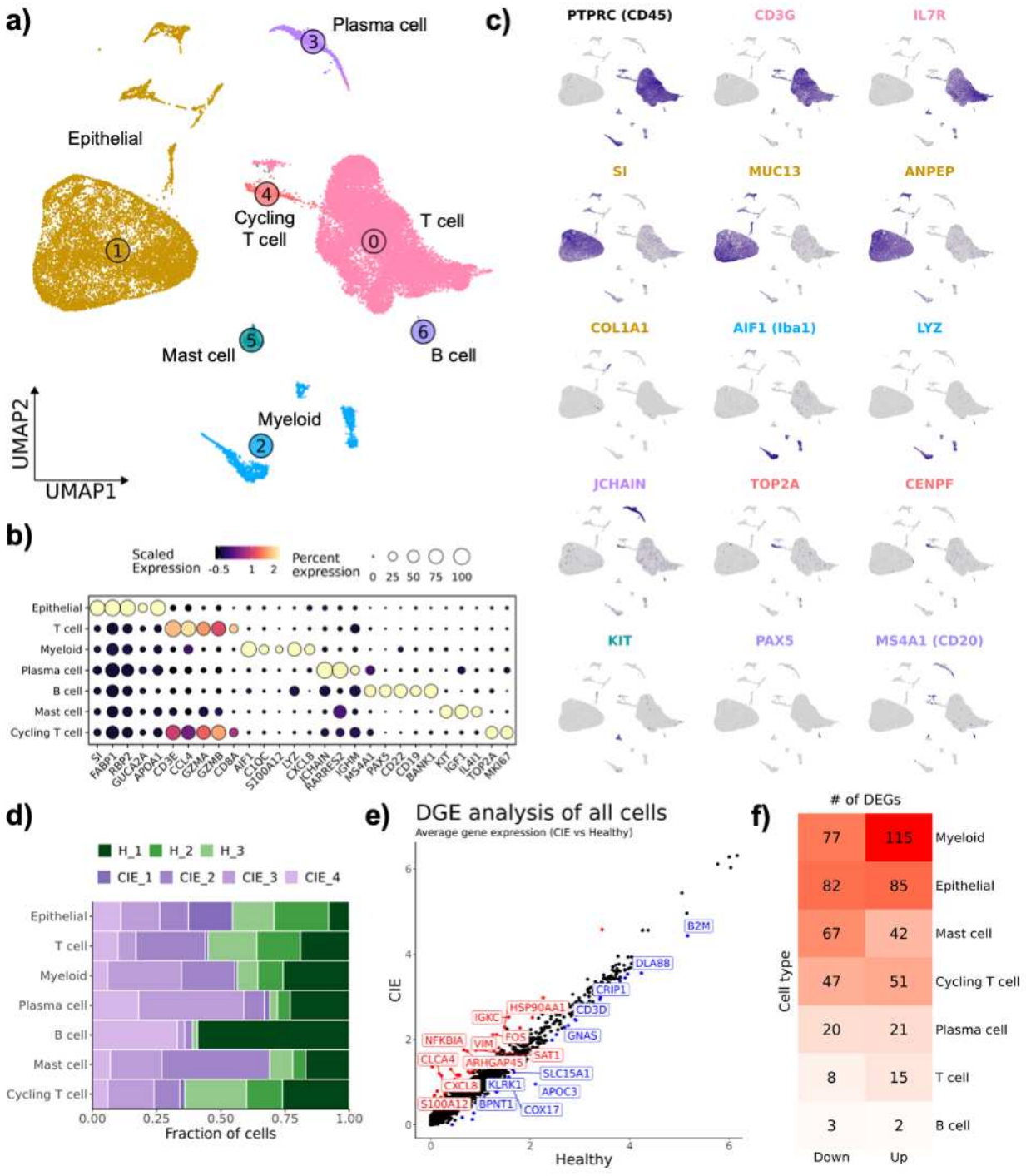
a)
Plasma cell
Epithelial
Cycling T cell
T cell
Mast cell
B cell
Myeloid
UMAP2
UMAP1
b)
Scaled Expression
Percent expression
Epithelial
T cell
Myeloid
Plasma cell
B cell
Mast cell
Cycling T cell
c)
PTPRC (CD45)
CD3G
IL7R
SI
MUC13
ANPEP
COL1A1
AIF1 (Iba1)
LYZ
JCHAIN
TOP2A
CENPF
KIT
PAX5
MS4A1 (CD20)
d)
H_1 H_2 H_3
CIE_1 CIE_2 CIE_3 CIE_4
Fraction of cells
e)
DGE analysis of all cells
Average gene expression (CIE vs Healthy)
CIE
Healthy
f)
# of DEGs
Cell type
Down
Up

Figure 5.1. Unsupervised clustering of duodenal endoscopic biopsies from 3 healthy and 4 CIE affected dogs separates cells into 7 distinct populations. (**a**) UMAP representation of 35,668 cells from dissociated duodenal biopsies from 7 dogs. (**b**) Dot plot representing scaled, log transformed gene expression for each major cell population. Dot size represents the percentage of cells expressing the gene while dot color represents relative expression. (**c**) Feature plots depicting expression of select genes associated with the identities of the major cell types. (**d**) Bar graph representing contributions to the 7 major cell populations by the 3 healthy dogs (greens) and 4 CIE dogs (purples). (**e**) Scatter plot depicting average gene expression in CIE (y-axis) compared to healthy cells (x-axis) with all 35,668 cells. Genes identified to have significantly different expression levels based on a Wilcoxon rank-sum test are colored red or blue. (**f**) Heatmap depicting the number of differentially expressed genes within each major cell type as determined using Wilcoxon rank-sum tests.

Neutrophilic Gene Signature Distinguishes Healthy From CIE Duodenal Mucosa

Analysis of AIF1 (Iba1) expressing cells revealed the presence of 6 myeloid cell subtypes: neutrophils, eosinophils, monocytes, macrophages, and 2 dendritic cell (DC) populations (Figure 5.2a/b, Table 5.2, Supplemental figure 5.3a, Supplemental data 5.4). Granulocytes (c0 and c1) were classified based on a lack of DLA-DRA (MHCII) expression while also expressing MS4A2 or S100A12 in the eosinophil and neutrophil clusters, respectively[44]. Monocyte, macrophage, and the two DC clusters were defined by DLA-DRA expression, with macrophages expressing MSR1 (CD204), monocytes lacking MSR1 expression, and DCs expressing FLT3[45]. Macrophages also exhibited preferential expression of CD163, CD64 (FCGR1A), and phagocytosis-associated genes (ANXA1, MER, NR1H2, NR1H3), features compatible with intestinal resident macrophages[46,47]. The FLT3 positive cells were divided into two populations, a conventional dendritic cell type 1 (cDC1) population[19] and a distinct DC population defined by expression of IL22RA2 and FSCN1. Statistical evaluation of myeloid cell subtype proportions revealed no statistically significant differences between the CIE and healthy dog samples (Supplemental figure 5.3b-d).

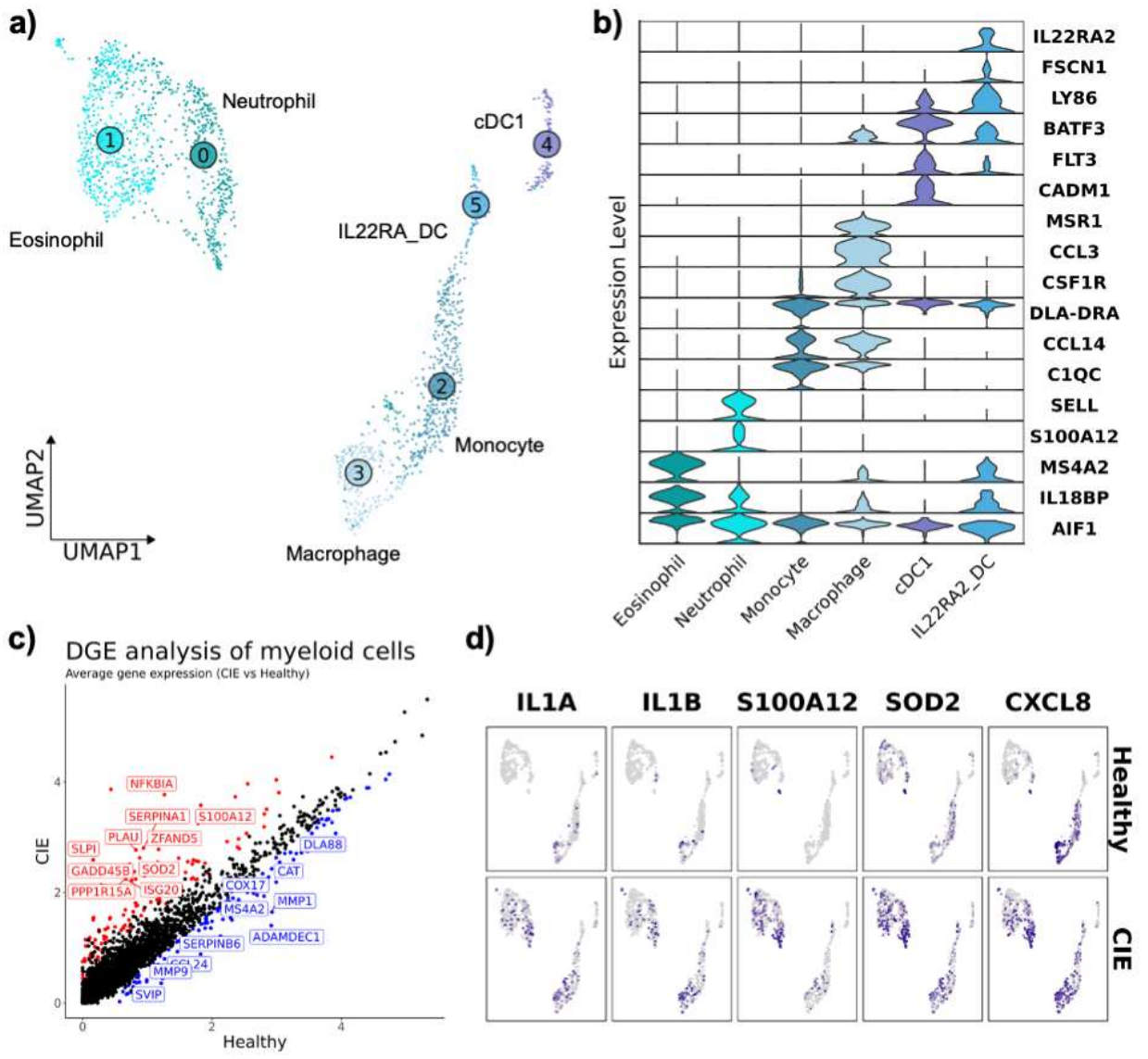

Figure 5.2. Subset analysis of myeloid cells highlights the presence of pro-inflammatory neutrophils in duodenal mucosa of CIE dogs. (**a**) UMAP representation of unsupervised clustering of 1,945 myeloid cells. Myeloid cell subtypes are labelled based on assigned identity, with increasing numbers corresponding to smaller relative contribution to the myeloid pool. Neutrophil (c0); eosinophils (c1); monocytes (c2); macrophages (c3); cDC1 (c4), conventional dendritic cell type 1; IL22RA_DC (c5); IL22RA expressing dendritic cells. (**b**) Violin plots of key features that define each subtype. (**c**) Scatter plot depicting average gene expression in myeloid cells isolated from CIE (y-axis) compared to healthy samples (x-axis). The top 10 genes identified to have significantly different expression levels based on Wilcoxon rank-sum test are labelled. (**d**) Split UMAP plots of select differentially expressed genes in duodenal myeloid cells from healthy (top pane) or CIE (bottom pane) dogs. High expression is indicated by dark purple coloring, while low expression is indicated by grey colorization.

Subsequently, we completed DGE analysis using a Wilcoxon rank-sum test to evaluate transcriptomic differences across all myeloid cells from healthy and CIE dogs. This which demonstrated a marked increase in the expression of genes related to inflammatory mediators (IL1A, IL1B, CXCL8, SOD2) in CIE affected dogs (Figure 5.2c). Gene set enrichment analysis (GSEA) of genes upregulated in CIE revealed an enhancement of pathways associated with interleukin signaling and neutrophil degranulation (Supplemental figure 5.3e), suggesting neutrophils may be influencing DGE analysis. Visualization of top DEGs in the UMAP space confirmed that most transcriptional differences between healthy and CIE dogs originated from increased expression within the neutrophil cluster, while also highlighting the role of monocyte/macrophage clusters (Figure 5.2d). This expression distribution suggests that duodenal mucosal neutrophils in CIE exhibit pro-inflammatory programs relative to those within the duodenal mucosa of healthy dogs. In summary, myeloid cells represented a small fraction (5.5%) of total duodenal mucosal immune cells in this dataset, but transcriptional differences suggest that pro-inflammatory neutrophils are enriched in the duodenum of dogs with CIE.

Subset Analysis of Duodenal T cells Reveals 10 cell subpopulations, Including Resident and Non-resident T cells

Through low-resolution unsupervised clustering of T cells (n = 18,824) we identified 4 transcriptomically distinct populations (annotated as tissue resident, non-resident, CCL4+ T, and innate lymphoid type 2 cells) (Figure 5.3a, Supplemental data 5.5). Clustering of T cells was not overtly driven by CD4/CD8 expression, as evidenced by varied expression of those features within each cluster (Figure 5.3b). Thus, we sought alternative means of distinguishing the T cell populations. Cells in the lower right portion of the UMAP exhibited overexpression of effector molecules (GZMA, GZMB), integrins (ITGB7, ITGAE), and FABP6 which is consistent with intestinal mucosal tissue-resident T cells[48,49,50].

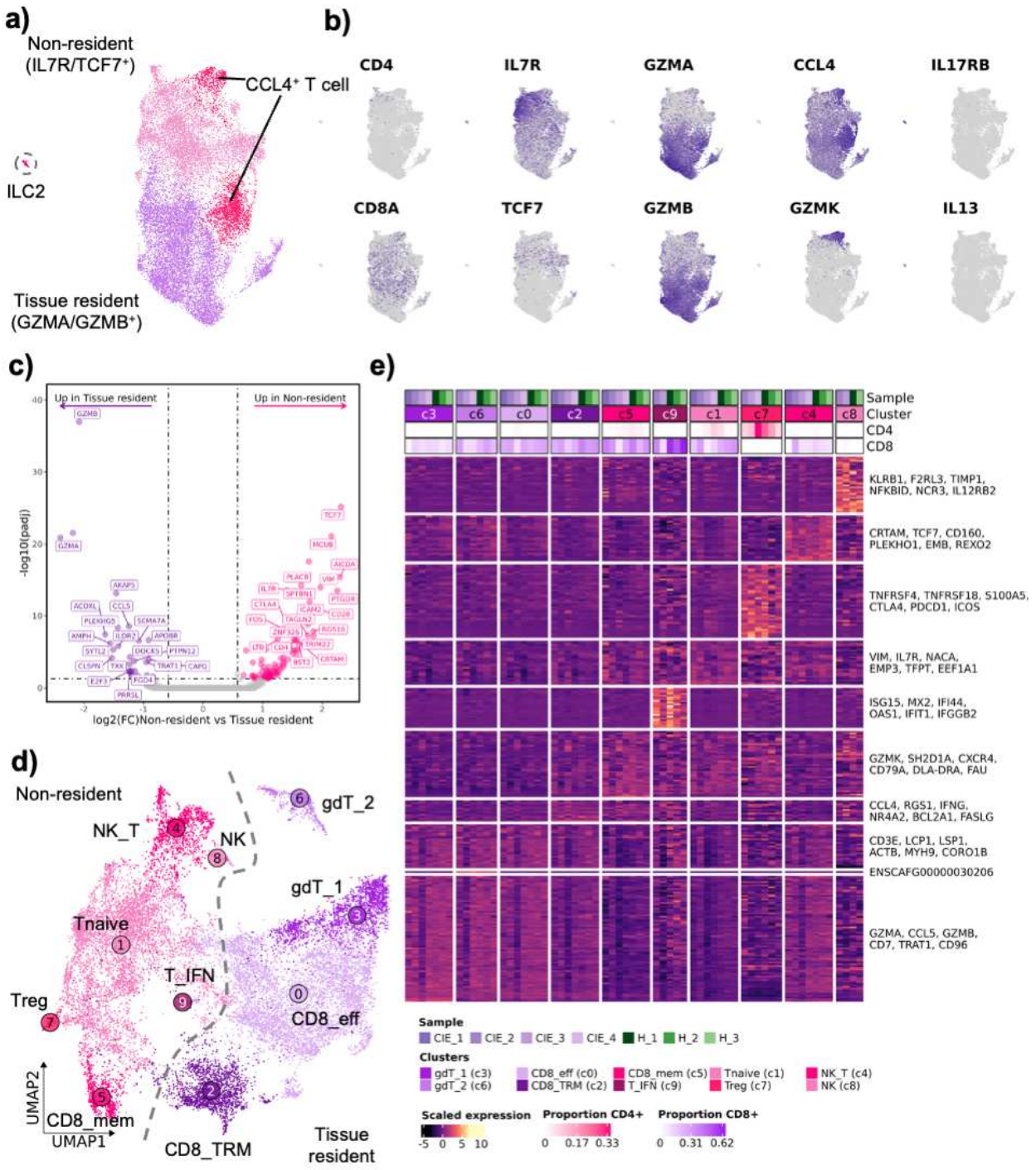
a)
Non-resident
(IL7R/TCF7+)
CCL4+ T cell
ILC2
Tissue resident
(GZMA/GZMB+)
b)
CD4
IL7R
GZMA
CCL4
IL17RB
CD8A
TCF7
GZMB
GZMK
IL13
c)
Up in Tissue resident
Up in Non-resident
-log10(padj)
log2(FC)Non-resident vs Tissue resident
d)
Non-resident
NK_T
NK
gdT_2
gdT_1
Tnaive
T_IFN
Treg
CD8_eff
CD8_mem
CD8_TRM
Tissue
resident
UMAP1
UMAP2
e)
Sample
Cluster
CD4
CD8
c3
c6
c0
c2
c5
c9
c1
c7
c4
c8
KLRB1, F2RL3, TIMP1,
NFKBID, NCR3, IL12RB2
CRTAM, TCF7, CD160,
PLEKHO1, EMB, REXO2
TNFRSF4, TNFRSF18, S100A5,
CTLA4, PDCD1, ICOS
VIM, IL7R, NACA,
EMP3, TFPT, EEF1A1
ISG15, MX2, IFI44,
OAS1, IFIT1, IFGGB2
GZMK, SH2D1A, CXCR4,
CD79A, DLA-DRA, FAU
CCL4, RGS1, IFNG,
NR4A2, BCL2A1, FASLG
CD3E, LCP1, LSP1,
ACTB, MYH9, CORO1B
ENSCAFG00000030206
GZMA, CCL5, GZMB,
CD7, TRAT1, CD96
Sample
CIE_1 CIE_2 CIE_3 CIE_4 H_1 H_2 H_3
Clusters
gdT_1 (c3)
gdT_2 (c6)
CD8_eff (c0)
CD8_TRM (c2)
CD8_mem (c5)
T_IFN (c9)
Tnaive (c1)
Treg (c7)
NK_T (c4)
NK (c8)
Scaled expression
-5 0 5 10
Proportion CD4+
0 0.17 0.33
Proportion CD8+
0 0.31 0.62

Figure 5.3. Subset analysis of duodenal T cells reveals 10 distinct cell subpopulations, including tissue resident and non-resident T cells. (**a**) UMAP representation of low-resolution unsupervised clustering of 18,824 T cells. Cells are divided into 4 broad categories based on the results of low-resolution unsupervised clustering: non-resident, tissue-resident, CCL4+ T cells, and type 2 innate lymphoid cells (ILC2s). (**b**) Feature plots displaying expression of select genes. (**c**) Volcano plot displaying differentially expressed genes contrasting non-resident to tissue resident T cell subpopulations based on DESeq2 analysis. The top 20 (by adjusted P values) differentially expressed genes are labeled. (**d**) UMAP representation of higher resolution unsupervised clustering of non-ILC2 T cells. (**e**) Heat map depicting scaled gene expression of the 10 T cell subtypes with individual dogs on columns and genes on rows. The proportion of CD4 or CD8 positive cells within each cluster is depicted in the top annotation of the heatmap. Non-resident T cells: [Tnaïve (c1), naïve T cells; NK_T, NK T cells (c4), CD8_mem, CD8 memory T cells (c5); CD8_mem, CD8 memory (Treg, regulatory T cells (c7); NK, natural killer T cell (c8); IFN_T, interferon signature T cells (c9)]. Tissue resident T cells: [CD8_eff, CD8 resident effector T cells (c0); CD8_TRM, CD8+ tissue resident memory T cells (c2); gdT_1, gamma delta T cell 1 (c3); gdT_6, gamma delta T cell 2 (c6)].

Through evaluation of features overexpressed in the top right portion of the UMAP, we determined the population to be compatible with naïve T cells based on enhanced IL7R and TCF7 expression[20]. Additionally, they exhibited enhanced CXCR4, SELL, and CCR7 expression, compatible with recently circulating, non-resident T cells[51,52]. To further compare these broad subdivisions of T cells, we completed DGE analysis to identify the features defining the two populations (Figure 5.3c, Supplemental data 5.6). Subsequent gene set enrichment analysis highlighted terms associated with immune cell differentiation and adhesion, further supporting the non-resident classification (Supplemental figure 5.4d). Unsupervised clustering further revealed the presence of a population of CCL4 expressing cells split between the tissue resident and non-resident regions of the UMAP (Figure 5.3a). This suggested that both non-resident and tissue resident cells have a subpopulation of memory cells expressing this T cell recruitment molecule[53]. The final major T cell population identified in our analysis was innate lymphoid type 2 (ILC2) cells. These cells clustered in a distinct region and were defined by IL13, IL17RB, and GATA3 expression[54,55]. To further corroborate the annotation of ILC2s, we completed refence mapping to the Human Gut Atlas, which confirmed the assigned identity as ILC2s (Supplemental figure 5.4a-c)[31].

With the potential for tissue-resident and non-resident subtypes to represent a biologically relevant subpopulations of T cells, we filtered out ILC2s and repeated subset

analysis at a higher resolution (Figure 5.3d, Supplemental figure 5.5a/b). This analysis revealed 6 subpopulations within the non-resident T cell parent population, including cells consistent with regulatory T cells (Tregs), natural killer (NK) cells, NK-T cells (NK_T), memory CD8 T cells (CD8_mem), T cells enriched in interferon gene signatures (T_IFN), and a population of naïve T cells (T_naive) (Table 5.2, Supplemental data 5.7). Within the tissue-resident parent population, an additional 4 subpopulations were identified which included two CD8 T cell populations annotated as CD8 tissue resident memory (CD8_TRM) and CD8 resident effectors (CD8_eff) (Table 5.2). Two groups of gd (gamma-delta) T cell subsets were annotated based on the expression of TRDC (gdT_1) or ENSCAFG00000030206 (TRGC2 ortholog) (gdT_2) (Supplemental data 5.7, Supplemental figure 5.5c).

To further evaluate the impact of CD4/CD8 expression in the clustering of T cells, we calculated the proportion of cells expressing CD4 or CD8 (normalized count > 0) within each cluster (Figure 5.3e). We found naïve T cells (c1) were composed of both CD4 and CD8 expressing cells, while Tregs (c7) had the greatest proportion of cells expressing CD4. Tissue resident T cells had a higher proportion of cells expressing CD8, although this was diminished in the gd T cell populations (c3/c6). While non-resident T cells had distinct cell type gene signatures, transcriptomic analysis of tissue resident T cells did not result in clearly defined cell subtypes, with substantial overlap in gene signatures (Figure 5.3e). This result suggests shared molecular programs between subtypes of tissue resident duodenal T cells. Lastly, while we were unable to detect any statistically significant T cell subtype abundance differences in CIE affected dogs, 2 of the 4 CIE dogs exhibited an overrepresentation of non-resident T cells (Supplemental figure 5.5d-f). Overall, results of our analysis provide a high-resolution depiction of T cells present in the canine duodenal mucosa and demonstrate limited evidence of CIE associated alterations in T cell subtype proportions and transcriptomic patterns.

Single-cell RNA Sequencing Reveals Marked Heterogeneity Among Duodenal Epithelial Cells

Investigation of the most abundant cell type, epithelial cells, revealed the presence of 4 enterocyte populations (c0-2, c6), BEST4$^+$ epithelial cells (c3), goblet cells (CLCA1$^+$/ARG2$^+$; c4), tuft cells (TRPM5$^+$/IRAG2$^+$/IL17RB$^+$; c5), enteroendocrine cells (CHGB$^+$/ADGRG4$^+$; c8), and a cluster of stromal cells (c7) (Figure 5.4a/b, Table 5.2, Supplemental figure 5.6a, Supplemental data 5.8). Cell type annotations were made using the Human Gut Atlas to complete reference mapping and gene signature enrichment (Supplemental figure 6b/c)[31]. Enterocytes (SI$^+$) were further annotated as 4 distinct populations with smallest cluster, IFN-enterocytes, being enriched in interferon-associated gene signatures (ISG15, RSAD2, IFIT1). The remining enterocyte clusters were divided based on the expression of solute carrier family members, where enterocyte 1 exhibited highest expression of amino acid transporters SLC22A4 and SLC43A2, enterocyte 2 had high expression of SLC40A1, whose role is in iron export, and enterocyte 3 was defined by expression of mitochondrial transporter SLC25A51 and anion transporter SLC25A6[56,57]. A small population of cells enriched in expression of genes associated with tissue structure and integrity (ACTA2, THY1, DCN, COL1A1) were classified as stromal cells[58]. Lastly, BEST4$^+$ epithelial cells were annotated based on the expression of BEST4, MT1E, GUCA2A, and CFTR which is consistent with the cell type identified in humans and are predicted to be involved in gut regulatory processes (e.g., motility, satiety) and the absorption of dietary heavy metals[26].

Next, we completed differential abundance analysis and found no statistically significant differences in cell subtype abundances between CIE and healthy dogs (Figure 5.4c), although a reduction in the proportion of Tuft cells (CIE = 1.84%, Healthy = 5.65%, *P* value = 0.057) was noted in dogs with CIE. Given the minimal shifts in cell type abundance we evaluated differential gene expression analysis to investigate disease associated transcriptomic differences. As

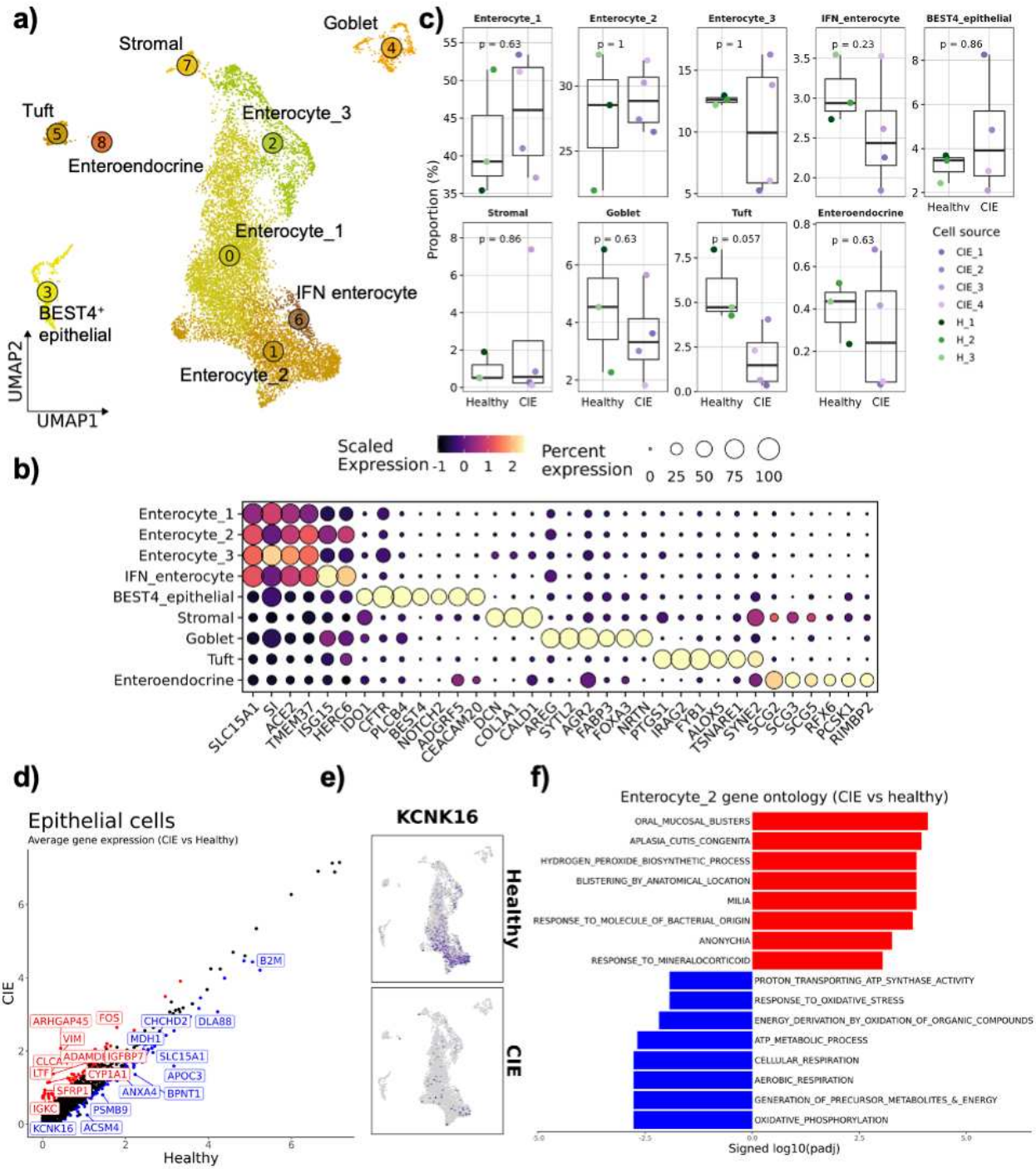
a)
Stromal
Goblet
Tuft
Enterocyte_3
Enteroendocrine
Enterocyte_1
IFN enterocyte
BEST4+ epithelial
Enterocyte_2
UMAP2
UMAP1
c)
Enterocyte_1
Enterocyte_2
Enterocyte_3
IFN_enterocyte
BEST4_epithelial
Stromal
Goblet
Tuft
Enteroendocrine
Proportion (%)
p = 0.63
p = 1
p = 1
p = 0.23
p = 0.86
p = 0.86
p = 0.63
p = 0.057
p = 0.63
Healthy
CIE
Cell source
CIE_1
CIE_2
CIE_3
CIE_4
H_1
H_2
H_3
Scaled Expression
-1 0 1 2
Percent expression
0 25 50 75 100
b)
Enterocyte_1
Enterocyte_2
Enterocyte_3
IFN_enterocyte
BEST4_epithelial
Stromal
Goblet
Tuft
Enteroendocrine
SLC15A1
SI
ACE2
TMEM37
ISG15
HERC6
IDO1
CFTR
PLCB4
BEST4
NOTCH2
ADGRF5
CEACAM20
DCN
COL1A1
CALD1
AREG
SYTL2
AGR2
FABP3
FOXA3
NRTN
PTGS1
IRAG2
FYB1
ALOX5
TSNARE1
SYNE2
SCG2
SCG3
SCG5
RFX6
PCSK1
RIMBP2
d)
Epithelial cells
Average gene expression (CIE vs Healthy)
CIE
Healthy
B2M
ARHGAP45
FOS
CHCHD2
DLA88
VIM
MDH1
CLCA
ADAMDEC1
IGFBP7
SLC15A1
LTF
CYP1A1
APOC3
SFRP1
ANXA4
BPNT1
IGKC
PSMB9
KCNK16
AC5M4
e)
KCNK16
Healthy
CIE
f)
Enterocyte_2 gene ontology (CIE vs healthy)
ORAL_MUCOSAL_BLISTERS
APLASIA_CUTIS_CONGENITA
HYDROGEN_PEROXIDE_BIOSYNTHETIC_PROCESS
BLISTERING_BY_ANATOMICAL_LOCATION
MILIA
RESPONSE_TO_MOLECULE_OF_BACTERIAL_ORIGIN
ANONYCHIA
RESPONSE_TO_MINERALOCORTICOID
PROTON_TRANSPORTING_ATP_SYNTHASE_ACTIVITY
RESPONSE_TO_OXIDATIVE_STRESS
ENERGY_DERIVATION_BY_OXIDATION_OF_ORGANIC_COMPOUNDS
ATP_METABOLIC_PROCESS
CELLULAR_RESPIRATION
AEROBIC_RESPIRATION
GENERATION_OF_PRECURSOR_METABOLITES_&_ENERGY
OXIDATIVE_PHOSPHORYLATION
Signed log10(padj)

Figure 5.4. Single cell RNA-seq shows marked heterogeneity within duodenal epithelial cell subtypes and marked reduced expression of KCNK16 in CIE mucosa. (**a**) UMAP representation of 12,457 epithelial cells. Epithelial cell subtypes are labelled based on identity, with increasing numbers corresponding to smaller relative contribution to the epithelial fraction. (**b**) Dot plot depicting gene expression in the 9 epithelial cell subtypes. Dot size represents the percentage of cells expressing the gene. (**c**) Box plots depicting relative abundances of epithelial cell subtypes between healthy and CIE dogs. P values were determined using a Wilcoxon rank-sum test. (**d**) Scatter plot depicting average gene expression in epithelial cells isolated from CIE (y-axis) compared to healthy samples (x-axis). The top 10 genes identified to have significant differential expression on a Wilcoxon rank-sum test are labelled. (**e**) Split UMAP plots depicting expression of KCNK16 in cells from healthy dogs (top pane) and from CIE dogs (bottom pane). (**f**) Bar chart representing results of gene set enrichment analysis comparing cells within the enterocyte_2 subtype from CIE dogs to those from healthy dogs (red bars indicate pathways overrepresented in CIE, blue bars indicate pathways overrepresented in healthy).

displayed in Figure 5.1f, we identified 62 upregulated and 78 downregulated features in CIE compared to healthy dogs using a Wilcoxon rank-sum test (Figure 5.4d). However, when we applied a more stringent DGE analysis approach (pseudobulk with DESeq2 workflow) we only identified 1 feature, KCNK16, to be downregulated in CIE (Supplemental figure 6e). To investigate the role of KCNK16 in this dataset, we visualized expression and identified the differential expression within the enterocyte 2 region of the UMAP (Figure 5.4e). Differential gene expression analysis and subsequent GSEA of the enterocyte 2 population identified induction of terms associated with mucosal blistering and scarring in cells from CIE dogs. Repression of terms associated with cellular respiration were also appreciated (Figure 5.4f), suggesting the enterocyte 2 cluster may be most impacted by the presence of CIE. Overall, our analysis identified marked diversity within canine duodenal epithelial cells and highlighted downregulation of KCNK16 in the enterocyte 2 cluster of CIE dogs, which may suggest a difference in the functional capacity of this enterocyte population in CIE.

5.5 Discussion

Chronic inflammatory enteropathy (CIE) is the most common reason for chronic diarrhea in dogs[2,3], yet its pathogenesis is poorly understood, preventing specific therapeutic interventions and evidence-based clinical management. The present study applied scRNA-seq to interrogate cell populations present in duodenal mucosal biopsies from healthy dogs and dogs with CIE. This approach enabled elucidation of the cellular diversity and transcriptomic programs present amongst cells of the canine duodenal mucosa. Analysis revealed disease-associated differences in neutrophil molecular programs and epithelial cell gene signatures while also highlighting diversity in T cell subtypes. Our analysis resulted in the generation of a reference dataset of canine duodenal cell type gene signatures that can be applied to further study the cellular and molecular mechanisms at play in chronic intestinal conditions affecting dogs.

Comparison of myeloid cells within the duodenal mucosa between the two groups of dogs revealed significant transcriptomic differences in duodenal neutrophils of CIE affected dogs. We found that cells isolated from CIE samples were the source of S100A12 and SOD2 overexpression identified through DGE analysis. This is consistent with findings of prior studies which have identified greater S100A12 expression in duodenal biopsies via ELISA[59] and higher S100A12 concentrations in fecal samples from dogs with CIE compared to healthy controls[60]. Other studies have identified the degree of neutrophilic inflammation in the lamina propria as a useful histopathologic parameter for CIE evaluation, showing a moderate correlation with CIE disease severity (CCECAI)[11]. Although our analysis did not identify a statistically significant increase in neutrophil relative abundances, GSEA results were indicative of increased neutrophil degranulation in CIE affected dogs. Given the limit number of myeloid cells in the dataset, further investigation in a larger cohort of dogs is required to elucidate the role of neutrophils, specifically the factors underlying their activation, in CIE pathobiology. Our results thus

underscore the benefit of gene expression profiling in tandem with comparison of cell proportions.

In line with results from IHC and flow cytometric evaluation of canine duodenal biopsies[38,61,13,62] we found $CD8^+$ T cells were the predominant T cell subtype. Nevertheless, canonical cell surface markers (CD4 and CD8) were generally expressed at very low levels and did not drive clustering. Instead, we found that T cells in our dataset were better discerned based on expression of genes indicative of tissue residency within the intestinal mucosa (tissue-resident T cells) or a more recently emigrated state (non-resident T cells). Based on gene expression profiles, we propose the non-resident T cells identified in our dataset correspond to T cells present within mucosal microvasculature at the time of biopsy, as well as T cells recently arriving in the duodenal mucosa from the blood stream. The cells classified as tissue resident T cells in the dataset presented here broadly expressed features associated with effector functions and integrins required for maintenance within mucosal tissue. While subclusters of non-resident T cells were readily identified, we found substantial overlap between tissue resident T cell subpopulations. This phenomenon of broad transcriptomic similarity among T cell subtypes has been previously described murine intestinal and airway T cells[63,64]. Our results shed light on the complex and heterogeneous intestinal T cell landscape beyond what has been identified using flow cytometric and IHC based analyses[38,14,38].

No statistically significant proportional differences were noted in T cells overall, or within T cell subtypes, between health and CIE. This is in line with results of previous studies unable to identify numerical increases in T cells in intestinal biopsies from CIE dogs compared to healthy ones[37,39,65] and contrasts a single study identifying greater numbers of intraepithelial and lamina proprial $CD3^+$ cells in duodenal biopsies from subsets of CIE dogs[14]. However, the control group in that study was selected based on having histologically “normal” intestinal biopsies, so this distinction could be arbitrary. Despite this, when we evaluated the proportions of tissue resident and non-resident T cells, we identified that two of four CIE affected dogs had an overabundance

of non-resident T cells with a concurrent reduction of tissue resident T cells in CIE duodenal mucosa compared to healthy. Precedent for shifts in T cell subtype proportions exist in celiac disease[21] and Crohn's disease[42], and so it is possible that with advanced methods of characterizing duodenal T cells, similar phenomenon may be uncovered in canine CIE. High interindividual variability and small sample size could have obscured a statistically significant difference. Future studies applying the T cell subtype gene signatures of the populations reported should be applied to larger populations of dogs to see if this observation is relevant to CIE pathobiology.

Through use of scRNA-seq, we were able to compare the transcriptomes of intestinal epithelial cells from healthy dogs and dogs with CIE. This minimally biased approach highlighted reduced expression of KCNK16, a gene encoding a 2-pore potassium channel within a subset of epithelial cells in CIE compared to healthy dogs. Potassium channels play essential roles in epithelial cell homeostasis, regulating membrane voltage, cell volume, and cell proliferation[66]. Furthermore, mice lacking the gene KCNK9 were found to be particularly susceptible to DSS-induced colitis[67]. Despite indications that the lack of duodenal KCNK16 expression may play a role in CIE pathogenesis, it is possible that the differential expression was due to breed differences, as our control population were all beagles and the CIE dogs consisted of 4 non-beagle purebred dogs. Further analysis of epithelial cells revealed KCNK16 downregulation was localized to a specific epithelial cell cluster (annotated as enterocyte_2) within the larger cluster of absorptive enterocytes. This epithelial cell type may represent the subset most affected by or responsible for CIE, and further investigation in the KCNK16 and the enterocyte_2 subpopulation is warranted.

Although the data reported here serves as a starting point for further investigation into the complex duodenal mucosa environment, the data and analyses performed here are not without limitations. Our endoscopic biopsies preferentially sampled superficial duodenal tissues (i.e., villus tips), which could have led to bias toward the intraepithelial cell populations, with

lesser contributions from the lamina propria. This may explain our inability to resolve Paneth cells, which are typically very rare cells in scRNA-seq analyses[26,68]. The tissue dissociation protocol and Ficoll density gradient centrifugation may have preferentially enriched for or depleted certain cell types[69,70], thus the proportions documented here may not exactly represent the proportions present in duodenal tissue in vivo. Furthermore, gene expression profiles of cells may have been influenced by the dissociation process, which has been reported to enrich for stress-response-related genes[70]. In our dataset, this was most evident within the enterocytes, which had higher-than-average percentage of mitochondrial reads. Lastly, we documented wide variability in cell type proportions isolated from individual CIE dogs with less variability among the 3 healthy beagle dogs. The heterogeneity observed within the CIE samples likely reflects the diverse pathogenesis of this condition in dogs, which is more accurately a syndrome rather than isolate disease entity. It is also possible that breed- and environment-specific differences may have contributed to observed differences between health and CIE. Comparison of healthy client-owned dogs of more diverse genetic and environmental backgrounds are needed to further advance understanding of canine gastrointestinal immunology.

The findings from this study provide important new insights into the pathogenesis of canine CIE, implicating intestinal epithelial and neutrophil molecular programs in disease. In addition, this work provides an important resource for the study of canine immune and GI cell types, as the dataset includes a comprehensive catalog of cells in the duodenum of dogs, which can be used to support future investigations.

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CONCLUDING REMARKS AND FUTURE DIRECTIONS

Chronic enteropathy in dogs is a highly prevalent and complex condition whose pathogenesis remains poorly understood. The projects described herein were embarked upon with the goal of improving the quality of life of dogs with CE. Within this context, we utilized *in vitro*, patient-based, and high-throughput approaches to investigate the pathogenesis of CE in dogs with an open-minded perspective. This is an exciting time in clinical medicine and immunology given the explosion of tools available to explore intricate systems like the GI tract.

Our investigation of bile acids' impacts on dog macrophages aimed to carry forward initial signals from untargeted metabolomic studies of dogs with CE[1] and humans with IBS and IBD[2,3]. We were able to document immunomodulatory effects of BAs on dog macrophages through cytokine and RNA sequencing analyses. Our findings represent the first functional readout on potential ramifications of much discussed BA dysmetabolism in dogs with CE[4,5,6]. As predicted based on data from in other species, primary and secondary BAs shift molecular activities of macrophages in dogs, with overlapping and contrasting impacts. However, many questions remain regarding the practical implications of our findings. For example, it remains unclear if "bile acid diarrhea" occurs in dogs, as is seen in up to a third of human IBS-D patients[7]. A few reports of dogs whose chronic diarrhea resolved when treated with a BA binder (e.g., cholestyramine) have generated excitement as a potential new therapy for CE dogs[8]. At this point, further work is needed to better elucidate the role of BAs in these patients. Our results suggest that deviations from a secondary BA (e.g., lithocholic acid) dominated distal gut BA profile could promote a pro-inflammatory environment. Still, our experimental model of canine macrophages in cell culture was several levels away from the complex gut environment. Next steps to explore the role of BAs more fully in dogs with CE could include more complex experimental set-ups involving different proportions of BAs to recapitulate the gut luminal and mucosal environment more accurately. Beyond gene expression and cytokine release, we also

need to evaluate the functional consequences of BAs on canine immune cell phagocytosis and antigen-presentation. Experiments utilizing canine jejunal and colonic organoids would allow for examination of the impacts of BAs on gut epithelial cells[9], which appear more central CE pathogenesis based on our initial transcriptomic data. Although it would be technically challenging, it would be ideal to repeat our experiment with canine macrophages isolated from intestinal biopsies, which appear to be phenotypically distinct compared to blood-derived monocytes[10]. Finally, attempting to corroborate Wang et. al.'s findings which related increases in fecal LCA concentrations with clinical improvement would be important to strengthen the theory that normalization of fecal BA profiles is an important therapeutic end point.

Diet is the most effective clinical strategy for treatment of dogs with CE, and this fact motivated the execution of a prospective clinical trial utilizing an amino acid based diet to treat dogs with CE, as described in chapter 3. We documented excellent palatability and positive clinical responses in line with results of other clinical trials utilizing diverse dietary strategies[11,12,13,14]. These results are encouraging as they point to another dietary option for dogs with CE, which will allow for expansion of the proportion of diet-responsive CE dogs. Based on our study, we would recommend a trial with EL prior to excluding the possibility of diet-responsive CE. More interesting, perhaps, than the clinical recovery we documented, are a few other aspects of that study. The dichotomy in fecal microbiota composition between diet responders and non-responders was unexpected. The ability of the diet to shift gut bacterial populations in some dogs appears to be an important factor which warrants further exploration. A logical next step in this direction could involve whole metagenomic sequencing on fecal samples. This would indicate biological functions inherent to the microbiota that are changed with dietary intervention, which may help provide better clues to the role of microbes in these dogs. To further characterize these dogs, we are in the process of analyzing baseline (T0) duodenal and colonic biopsies with bulk RNA-seq to see if gene signatures distinguish these groups of dogs and provide clues to why some does improve with diet and others are refractory.

However, post-treatment biopsies may be necessary to comprehensively explore this question. A non-invasive means of monitoring GI disease in dogs with CE would represent a major step forward, as serial endoscopies are expensive and involve some patient risk. This has been an ongoing project of our research team. The end goal is a test that would obviate the need for intestinal biopsies through transcriptomic profiling of exfoliated host mRNA (exfoliome). This may be a lofty goal, but the potential pay-off to patients, their families, and veterinarians makes this endeavor worth the effort.

The same week that our clinical trial using the amino acid based diet was published, Simpson et. al. published findings from a clinical trial utilizing diets for remission of CE in client-owned dogs[15]. Their group documented achievement of clinical remission in 19/23 normalbuminemic CE dogs with ingestion of 1 of 3 diets. They identified no difference in efficacy between the diets sourcing protein from hydrolyzed fish, nonhydrolyzed fish or chicken, leading them to hypothesize that positive responses may not be driven by antigen restriction. These results provide important context in which to consider the results of our clinical trial, whereby the provision of protein via individual amino acids may not be the most important aspect of why dogs improved. This is in line with results from pediatric CD patients treated with EEN, whereby polymeric formulae are not inferior to elemental ones[16]. In any case, diet remains the most powerful tool in the arsenal against CE, and by disentangling its mechanisms of action, more dogs will be able to achieve relief from their disease.

Clinical trials investigating CE dogs will continue to be central to study of this disease. A key concept going forward, particularly with the types of in-depth analyses we are completing, involves characterizing clinical patients are specifically as possible. Years of study of CE have had their power curtailed by blurred lines between PLE and CE dogs. Moreover, specific clinical characteristics need to be included as metadata for CE studies, so that in the future it might be possible to tease apart disease subtypes. This must go beyond assignment of a CIBDAI/CCECAI score and a treatment response category. We could draw inspiration from

questionnaires developed for humans with IBS to extract more details regarding GI patterns in sick and healthy dogs, which will increase the power of patient-based analyses.

The results from our bulk RNA-seq comparison of duodenal mucosa in health and in CE in chapter 4 provide a foundation from which to further explore the molecular mechanisms which are disrupted in CE. Before any major conclusions are drawn, we need to increase our sample size. Still, with this initial dataset, our approach highlighted discrepancies between patients that would not have been predicted and have not been previously explored. Exploration of IEC dysfunction needs to be pursued, as most of the top DE genes mapped back to IECs. Moreover, it may be worth revisiting the presence of viral co-infections in CE patients, given the pathway analysis highlighting upregulation of RIG-I and IFN pathways. Our results encourage a more open-minded approach to CE – immune suppression may be contraindicated in CE dogs (or a subset of them) if they are struggling to deal with viral pathogens. Going forward, we are keen to integrate results of other omics analyses to our gene expression profiling. Our initial plan was to sequence bacterial DNA from concurrently collected biopsies and generate correlations, however, the bacterial sequence data was not usable from enough dogs. For our next study, we will flash freeze a separate set of biopsies to maximize adherent bacterial DNA yield.

In chapter 5, we applied scRNA-seq to catalog the cells present in duodenal biopsies from CE and healthy dogs. This approach allowed us to characterize the cellular makeup of the intestinal mucosa to a degree not previously accomplished in dogs. Many novel conclusions were drawn from this project. We are motivated to further explore the role of duodenal neutrophils in CE. As a starting point, it would be interesting to see if expression of the genes associated with infiltrative neutrophils in our patient population (SOD2, CXCL8) correlate with disease severity and/or identify patients with distinct CE subtypes in a larger patient population. Investigations into the factors driving neutrophil infiltration and activation in the GI mucosa are also needed. Additionally, we are curious regarding the apparent depletion of KCNK16 expression in CE compared to healthy tissues. We intend to collect tissues from healthy non

beagle dogs and quantify expression of this gene to determine if it is truly different in health vs. CE. Given the skew toward expansion of non-resident T cells and relative depletion of tissue resident T cells in CE duodenal mucosa, further exploration of intestinal epithelial and LP T cells is warranted. On a broader scale, the gene signatures we have established with this dataset provide a platform for the development of more clinically accessible assays. Validation will need to be performed to see if a panel of genes can recapitulate the cellular identities indicated by scRNA-seq. Further investigation of the functional capacities of the cell subtypes documented in this study would also be important. Flow sorting of dissociated biopsies would allow for this, and *in vitro* assessment of antigen processing and presentation capabilities, as well as cytokine production, could be performed to elucidate T cell subtype behaviors. Given the vast amounts of information gleaned from this initial scRNA-seq study, we are motivated to perform similar analysis on ileal and colonic biopsies so that these canine databases exist. Then, we can move forward with bulk RNA-seq based studies which will be much more affordable and efficient.

In summary, results of the studies described here has added much needed insight into various aspects of chronic enteropathy in dogs. Our findings further underscore the vast complexity of the gastrointestinal system, making it difficult to believe that for most individuals, most of the time, things work out well. As with most research endeavors, completion of one project motivates completion of additional experiments and trials to answer newly uncovered questions. Our work provides a solid foundation for further studies to be completed to add more pieces to the puzzle of the amalgam that is canine CE. We can at once be humbled regarding our limited understanding and inspired by available technologies that offer possibilities of disentangling the roles of host immune and epithelial cells, in concert with commensal microbes and local metabolites.

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