IDENTIFICATION OF NOVEL BIOMARKERS

IN CATS WITH CHRONIC ENTEROPATHIES


A Dissertation

by

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ABSTRACT


Feline chronic enteropathy is a very common disorder with an increasing

prevalence over the past decades. It mainly comprises idiopathic inflammatory bowel

disease (IBD) and alimentary small cell lymphoma (SCL). Histopathology,

immunohistochemistry, and clonality testing are currently considered to be the gold

standard for the diagnosis and differentiation of IBD from SCL. The performance of

these tests in a cohort of clinically healthy client-owned cats with similar demographic

characteristics was evaluated. To characterize the mucosal proteome of cats with chronic

enteropathy and to identify novel biomarker candidates, two-dimensional fluorescence

difference gel electrophoresis was performed on intestinal biopsies. To characterize the

fecal microbiome and metabolome, 16S rRNA sequencing and ultra-performance liquid

chromatography were performed on fecal samples from cats with chronic enteropathy.

Histopathology, immunohistochemistry, and clonality testing in healthy cats

frequently revealed findings that are considered abnormal based on the currently

accepted standards. Tests that are currently considered to be the gold standard for the

diagnosis of feline chronic enteropathy should be interpreted with caution. Several

potential protein biomarkers were identified in the intestinal mucosa of cats with CE.

Among the identified proteins were those of the annexin and apolipoprotein families,

and malate dehydrogenases. Characterization of the fecal microbiome of cats with

chronic enteropathy revealed a dysbiosis pattern that has previously been described

across different species with intestinal inflammation. The dysbiosis was characterized by

a significantly reduced alpha diversity and trends for increased facultative anaerobic bacteria (e.g., *Enterobacteriaceae*) in favor of obligate anaerobic bacteria. However, the overall dysbiotic pattern did not differ between cats with IBD and cats with SCL.

Characterization of the fecal metabolome revealed global metabolic changes in cats with chronic enteropathy with many pathways involved such as the tryptophan pathway, amino acids, sphingolipids, and sterols. However, the global metabolic pattern did not differ between cats with IBD and SCL. Further work is needed to verify these findings and confirm their promise for the development of a clinically useful biomarker or biomarker panel for the definitive diagnosis and sub-classification of feline chronic enteropathy.

DEDICATION

I dedicate this work to my mother Ilse Marsilio (née Wilke) and my father

Casimiro Marsilio. My mother's tenacity, devotion and discipline and my father's

enthusiasm and fierceness were inspirational to me.

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**Contributors**

This work was supervised by a committee consisting of Dr. Jörg M. Steiner (advisor), Dr. Jan S. Suchodolski (co-chair), and Dr. Jonathan A. Lidbury, all from the Department of Small Animal Clinical Science, Dr. Lawrence J. Dangott from the Department of Biochemistry and Biophysics, and Dr. Rachel Rech from the Department of Veterinary Pathobiology.

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NOMENCLATURE

2D DIGE      two-dimensional fluorescence difference gel electrophoresis

CD      cluster of differentiation

CE      chronic enteropathy

FFPE      formalin-fixed, paraffin-embedded

fPLI      pancreatic lipase immunoreactivity

fTLI      feline trypsin-like immunoreactivity

H&E      hematoxylin and eosin

IBD      idiopathic inflammatory bowel disease

LC-MS/MS      liquid chromatography tandem-mass spectrometry

MS      mass spectrometry

NK      natural killer

PARR      PCR for antigen receptor rearrangements

SCL      small cell lymphoma

SI      small intestinal

TRG      T cell receptor gene

UPLC      ultra-performance liquid chromatography®

WSAVA      World Small Animal Veterinary Association

TABLE OF CONTENTS

LIST OF FIGURES

# 1. INTRODUCTION

## 1.1. Importance and background

Feline chronic enteropathy (CE) is a spontaneously arising disorder in cats that is especially common in the elderly cat population. It is defined as the chronic presence (i.e., longer than 3 weeks) of signs of gastrointestinal disease such as weight loss, vomiting and diarrhea in the absence of extraintestinal causes[1].

The disorder mainly comprises idiopathic inflammatory bowel disease (IBD) and alimentary small cell lymphoma (SCL). While the etiopathogenesis of IBD is unknown, it appears that, similar to IBD in people, genetic predispositions, environmental factors, the gastrointestinal microbiota, and aberrations in the immune response all play a role[1,2]. In addition, it has been anecdotally reported that chronic inflammation in cats with IBD may trigger neoplastic transformation and thus that IBD may progress to SCL in cats[3-5]. However, longitudinal studies to investigate this hypothesis are currently lacking. Diagnosis and differentiation can be challenging as clinical signs might be the same in both disease entities[6]. Currently, the diagnosis of both, IBD and SCL, is based on histopathologic examination of gastrointestinal biopsy specimens acquired under general anesthesia[7]. Hence, the diagnosis of IBD and SCL is elaborate, expensive, time- and resource-consuming, and highly-invasive. Treatment of IBD and SCL is usually based on immunomodulation by various corticosteroids or cyclosporine, or even cytotoxic medication, such as chlorambucil[8,9]. Therefore, less invasive biomarkers for diagnosis of feline CE would be highly desirable.

**1.2. Feline chronic enteropathy**

The term chronic enteropathy describes a group of diseases that cause chronic gastrointestinal signs including the following entities[10,11]:

1. Food-responsive enteropathy (FRE)

2. Antibiotic-responsive enteropathy (ARE)

3. Idiopathic inflammatory bowel disease (IBD)

4. Alimentary small cell lymphoma (SCL)

After infectious and dietary causes as well as extra-gastrointestinal diseases have been excluded as causative factors, the main differential diagnoses for feline CE are idiopathic inflammatory bowel disease (IBD) and alimentary small cell lymphoma (SCL).

However, differentiating IBD from alimentary SCL remains a challenge for the clinician and the pathologist[12].

**1.2.1. Signalment**

Feline CE usually occurs in middle-aged to older cats. Although, cats with idiopathic IBD tend to be younger (median or mean age 7.7 – 9.7 years, range: 1.3 - 16)[6,10] than cats with SCL (median or mean age 12.3 to 13 years, range: 4 – 20 years)[6,8,9,13,14] there is considerable overlap between populations[6-10,13]. However, SCL appears to be uncommon in cats below the age of 6 years. Male cats and Siamese or other Asian breeds have anecdotally been described to be predisposed, however, this has not been a consistent finding[1,10,15].

**1.2.2. Clinical signs**

The most common clinical sign of cats with feline chronic enteropathy is weight loss (80 - 90%), followed by vomiting (70 - 80%), hyporexia (60 - 70%), and diarrhea (50 - 83%)[4,6,8-10,16-18]. While some authors report cats with FRE to be younger and having more frequent diarrhea with other clinical signs being less common[19], this has not been confirmed by others[10]. Common findings on physical examination are a low body condition score, sarcopenia, thickened bowel loops, and/or abdominal pain[19,20]. However, physical examination may also reveal no abnormal findings. Similar to dogs, cats with FRE might suffer from cutaneous lesions including itchiness and alopecia[1]. Abdominal mass lesions or effusion are usually associated with high grade lymphomas, which usually shows a rapid clinical course[6]. In contrast, clinical signs in cats with idiopathic IBD and SCL are usually slowly progressive over weeks to months and often cyclic[1,4,6,10].

After extra-gastrointestinal diseases (e.g., hyperthyroidism, advanced chronic kidney disease, cancer etc.), infectious intestinal diseases (e.g., helmiths, *Tritrichomonas foetus* or other parasites etc.), and dietary hypersensitivity have been excluded, the main differential diagnoses for CE are idiopathic IBD or alimentary SCL. Clinical signs are virtually the same and the collection of biopsies is necessary for the definitive diagnosis and differentiation of feline IBD from SCL[6].

### 1.2.3. Idiopathic inflammatory bowel disease

The term idiopathic IBD is reserved for patients in which all (currently) known causes for gastrointestinal signs have been excluded and histopathologic examination of gastrointestinal biopsies demonstrates mucosal inflammation with no etiological agents and thus, the disease can be assumed to be idiopathic[1,16,21]. Based on the dominant mucosal infiltrate, different forms of inflammatory bowel disease, such as lymphocytic plasmacytic IBD, eosinophilic, granulomatous and suppurative IBD, can be differentiated[21-23]. Lymphocytic plasmacytic IBD is by far the most common form of IBD, while other forms are less commonly idiopathic and more often associated with an underlying disease pathology[23]. Besides the mucosal infiltration with inflammatory cells, mucosal architectural changes are common findings in tissue biopsies from cats with idiopathic IBD[24]. In 2008, the World Small Animal Veterinary Association (WSAVA) described histopathological standards for the diagnosis of gastrointestinal inflammation in endoscopic biopsy samples from cats and dogs [24,25]. The establishment of the WSAVA standardization criteria was the professions response to the previously reported, high inter-observer variability between different pathologists[26]. The WSAVA histopathological standards include the description of normal and abnormal findings in the stomach, duodenum and colon. Pictorial templates and scoring sheets, assessing cellular infiltration and morphological changes intend to assist the pathologist in establishing a more objective diagnosis. The WSAVA standard form for the assessment of duodenal mucosa can be found in Table 1.1

Table 1.1. WSAVA standard form for assessment of duodenal mucosa

Pathologist          Case Number

Number of pieces of duodenal tissue on slide_____

Tissue present

 Inadequate        Too superficial   Adequate depth

Number of tissues abnormal _____

| | **Morphological features** | | | |
|---|---|---|---|---|
| | Normal | Mild | Moderate | Severe |
| Villus stunting | | | | |
| Epithelial injury | | | | |
| Crypt distention | | | | |
| Lacteal dilatation | | | | |
| Mucosal fibrosis | | | | |

| **Inflammation** | | | | |
|---|---|---|---|---|
| Intraepithelial lymphocytes | | | | |
| Lamina propria lymphocytes and plasma cells | | | | |
| Lamina propria eosinophils | | | | |
| Lamina propria neutrophils | | | | |
| Others | | | | |

| **Final diagnosis** |
|---|
| Normal tissue |
| Lymphoplasmacytic inflammatory |
| Eosinophilic inflammatory |
| Neutrophilic inflammatory |
| Lymphangiectasia |
| Mucosal atrophy/ fibrosis (non-inflammatory) |
| Others |
| Other comments |

However, despite the attempt to standardize the histopathological assessment of gastrointestinal tissue biopsies, inter-observer variability remains a concern[27]. Other limitations of the WSAVA standardization criteria are related to the included population of cats used to develop it. The guidelines were established mainly based on full-thickness biopsies of relatively young cats between the ages of 5 and 18 months that were kept under specific pathogen free (SPF) conditions[28,29]. This is in contrast to the population of cats with CE, which comprises commonly middle-aged to older cats[4,14]. In addition, the standards were developed for the assessment of intestinal inflammation in dogs and cats, while cats with SCL where not included in the guidelines. However, clinically as well as histopathologically SCL is the most important differential diagnosis for feline IBD. These limitations call the applicability of the WSAVA standards to the population of cats with CE into question. The work of this dissertation is the first to describe histopathological findings in endoscopically obtained gastrointestinal tissue biopsy specimens in a cohort of healthy, client-owned pet cats with similar demographic characteristics as cats presented for CE.

**1.2.4. Feline alimentary lymphoma**

Lymphomas are characterized by a clonal expansion of one (or sometimes few) lymphocyte clones[30]. This clonal cell population is mostly either of T-cell origin or B-cell origin[31]. Gastrointestinal lymphomas can be classified by morphologic evaluation (small vs. large cell, distribution within the mucosa, epitheliotropism) immunophenotyping (B-cell, T-cell, NK-cell), anatomical location, and its clinical behavior (low grade vs. high grade)[31]. From a clinical as well as pathological

perspective, three main types of feline gastrointestinal lymphoma can be differentiated, that differ in appearance, treatment, and prognosis: 1) low grade, small cell lymphomas 2) intermediate to high grade, large cell lymphomas, and 3) large granular lymphocyte (LGL) lymphomas[31].

Intermediate and large cell lymphomas represent a progressively less differentiated, more immature cell population[31]. Mitotic rates are higher and they are more aggressive tumors, following a more acute and rapid clinical course. Large granular lymphocyte lymphoma is a separate form of gastrointestinal lymphoma in cats, which may show variable cell size, but is generally considered to be a high grade, aggressive neoplasm[32]. From a clinical perspective, these differentiations are important as signalment, clinical findings, biologic behavior, therapy and, most importantly, prognosis significantly differ between the different forms of gastrointestinal lymphoma[8,13,33].

Small cell lymphomas (SCL) are the most common form of alimentary lymphoma in cats accounting for up to 75% of all gastrointestinal lymphomas in this species[31]. They consist of small lymphocytes that represent a mature, well-differentiated population of cells with low mitotic rates[8,34]. Clinical progression is usually slow, with clinical signs often present over many weeks to month[35-37]. Thus, these lymphomas are classified as low grade, small cell lymphomas[9]. Over 90% of small cell lymphomas are of T-cell origin and they usually arise in the jejunum, duodenum, or ileum in descending order of frequency of occurrence[31]. Gastric or colonic SCL are uncommon[31]. Small cell lymphomas commonly cause diffuse infiltration and thus diffuse thickening of the

affected small intestinal segment(s) and are often limited to the mucosa, but may also occur transmurally[31]. Several authors have compared feline alimentary small cell lymphoma to monomorphic epitheliotropic intestinal T-cell lymphoma (MEITL) in humans (previously called enteropathy-associated T-cell lymphoma Type 2, EATL Type 2)[12,31]. However, while alimentary lymphoma is a relatively indolent, slowly progressive disease, the prognosis for MEITL is considered poor because of the rapid clinical course and the resistance to treatment[38]. More recently, other authors suggested that the cat might be a suitable model for indolent digestive T-cell lymphoproliferative disorder[17]. This rare disorder in people is characterized by a superficial monoclonal intestinal T-cell infiltrate with an indolent or slowly progressive clinical course[17,39-43]. Thus, it appears that there are some parallels between both disease in humans and cats.

SCL can be difficult to distinguish from inflammatory infiltrates found on intestinal biopsies or fine needle aspirates in patients with other chronic enteropathies such as IBD[12,31]. Both entities are characterized by mucosal infiltration with small, mature lymphocytes that exhibit low mitotic rates[19,20]. In addition, concurrent inflammatory lesions have been described in up to 60% of cats with alimentary lymphoma[12,31,34,36,44]. It has been hypothesized that chronic inflammation might promote neoplastic transformation and that IBD in cats may progress to SCL over time[4,14,34,45].

Among histopathological criteria for the differentiation of SCL from idiopathic IBD are epitheliotropism and transmural infiltration of the intestinal wall, both of which are usually only seen in alimentary lymphomas. Epitheliotropism describes the arrangement of neoplastic T-cells within the mucosal epithelium in form of nests and

plaques[12,31,46]. Intraepithelial nests are defined as 5 or more small T-cells clustered

within the villus epithelium, while intraepithelial plaques refer to 5 or more adjacent

epithelial cells obscured by infiltrates of small T-cells[12]. However, two different studies

found that the majority of intraepithelial cells in healthy and SPF cats were CD3+ T-

cells [28,29], and thus the presence of intraepithelial small T-cells is not always indicative

for SCL. Conversely, the absence of intraepithelial T-cells does not exclude SCL since

not all SCLs show epitheliotropism[12]. Therefore, further diagnostics are frequently

required and include immunohistochemistry of intestinal biopsies with staining for CD3,

expressed by T-cells and/or CD79a, CD20, or PAX-5 expressed by B-cells[4,12]. In

addition to immunohistochemistry, the clonality of the lymphocyte population can be

assessed using PCR for antigen receptor rearrangements (PARR)[12,31,34]. Lymphomas

consist of a clonal or oligoclonal cell population, while inflammatory lesions are

generally polyclonal in nature with a variety of lymphocyte clones with different antigen

receptors being recruited to the site. However, data in human patients with

lymphoproliferative disease shows, that while the sensitivity of PCR-based clonality

assays is generally high (> 90%), the specificity for T-cell receptor rearrangements

might be as low as 54.3% in reactive lesions[47]. While the sensitivity and specificity of

PCR-based clonality assays in veterinary medicine is unknown, it is unlikely to be

higher than that described in human medicine where assays, including the assay

interpretations, are standardized[48,49]. Therefore, less invasive and more reliable

biomarkers for the diagnosis and differentiation of feline CE are warranted.

**1.3. Biomarkers**

In 2001, the Biomarkers Definitions Working Group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"[50].

According to the Federal Drug Administration[51] an ideal biomarker should have the following characteristics:

- sensitive and specific for a disease and able to differentiate between different physiological states

- non-invasive and accessible to measure

- rapid so as to enable faster diagnosis

- economical

- able to give accurate results

- consistent between different demographic groups

The FDA also defines seven different categories of biomarkers[51]:

- susceptibility/risk biomarker

- diagnostic biomarker

- monitoring biomarker

- prognostic biomarker

- predictive biomarker

- pharmacodynamic/response biomarker

- safety biomarker

The process of biomarker development comprises four main steps: discovery, analytical validation, evaluation of clinical utility, and clinical application. In the discovery phase, the analysis of biological specimens, such as tissue or feces leads to the identification of candidate biomarkers[52]. The advent of high-throughput processes underpinning "-omics" technologies is now contributing to the discovery of novel biomarkers as multiple markers can be screened in parallel[53].

An important consideration when investigating IBD biomarkers is that a single marker may not provide the clinical utility desired, whereas the same biomarker may have utility when combined with others in a panel governed by a scoring index or algorithm[54].

### 1.3.1. Biomarkers previously investigated for feline chronic enteropathy

Previous studies investigated the use of laboratory parameters such as serum albumin[18,45], serum total protein[10], serum cobalamin[9,18,55,56], serum folate[18], serum ALT[6,10], serum lactate-dehydrogenase[57], and fecal α1-proteinase inhibitor concentration[18] for the diagnosis and differentiation of feline idiopathic IBD and SCL. However, none of these laboratory markers revealed to be clinically useful for the differentiation of feline idiopathic IBD from SCL due to large overlap in results between groups. Similarly, imaging-based techniques have been investigated for their usefulness of CE in cats[58,59]. Although, older cats with ultrasonographic thickening of the tunica muscularis were more likely to have SCL than IBD, ultrasonographic patterns were not pathognomonic and overlap between the diseases was found[58].

### 1.3.2. Omics-based biomarker discovery

Omics technologies are high-throughput techniques that allow for the assessment of large sets or subsets of the genome, transcriptome, proteome, metabolome, microbiome etc.[52,60]. They are powerful tools for the discovery of new, disease specific biomarkers by analyzing and comparing large datasets between different individuals with a specific condition often generating thousands of data points at the same time[52,61]. However, the disadvantage of most omics technologies is a high false discovery rate and the lack of quantification[52]. Therefore, biomarker candidates identified by high-throughput technologies have to be validated by other analytical platforms during the qualification and verification phase[62].

### 1.3.3. Biomarkers and the proteome

The proteome is the complete set of proteins expressed by a cell, tissue, or organism[63]. Although, the proteome is an expression of an organism's genome, the proteome dynamically changes in response to intrinsic and extrinsic factors, such as various disease states[63]. This is in contrast to the genome, which is characterized by its relative stability. The study of proteomics involves large scale detection, identification, and characterization of proteins, making it highly promising for biomarker discovery across many diseases.[64] The most common method applied is a combination of two-dimensional gel electrophoresis (2-DE) and mass-spectrometry. 2-DE provides a powerful tool for isolating proteins that differ in abundance between cases and controls. Following 2-DE, mass spectrometry is used to identify differentially expressed proteins.[64]

12

The field of proteomics is a rapidly expanding area of research that has been employed in many diseases including IBD and cancer in humans.[65] Proteomic studies were able to identify various potential biomarkers in people with IBD from serum or intestinal tissue samples such as ceruloplasmin, clusterin, and apolipoprotein B-100.[65] Since biomarkers are usually among the low-abundance proteins, the concentration of candidate IBD biomarkers may be higher in intestinal tissue compared to serum. Thus, using tissue samples in the discovery phase might be advantageous and potentially reduces the chance of false discovery[66,67]. Once a protein has been identified as a potential biomarker, a targeted approach to detect this protein in other samples such as blood, urine, or feces can be undertaken[66,67].

To the author's knowledge, no data on the proteome in cats with IBD or SCL have been published.

**1.3.4. Biomarkers and the microbiome**

In recent years our knowledge about the role and complexity of the GI microbiota has substantially increased. Intestinal microbes have been shown to modulate the host's immune system within and beyond the gastrointestinal tract and to act as a defense mechanism against various pathogens[68]. Many changes in both the structure and function (i.e., metabolome) of the gut microbiota are associated with IBD. In humans with IBD, a dysbiosis with a generally decreased biodiversity has been found.[69,70] Dysbiosis has also been described in cats and dogs with acute and chronic GI disease.[71-75]

Specific changes of the microbiota in human and canine IBD are characterized by increases in the proportions of bacterial genera belonging to *Proteobacteria* and decreases in *Fusobacteria*, *Bacteroidetes*, and members of the *Firmicutes* (e.g. *Faecalibacterium spp., Turicibacter spp., Blautia spp.*).[72,73,75,76] Similar observations have been made in a study utilizing 16S rRNA sequencing in cats with chronic diarrhea.[71]

These changes can sustain and potentially trigger IBD, promote complications and contribute to poor response to treatment.[75] The inflammatory milieu in IBD selects against commensals that are capable of survival and growth in this environment and many of the microbes that are more abundant in patients with IBD have pro-inflammatory properties, for example *Bilophila wadsworthia*.[77] However, these complex and often subtle changes are difficult to apply to routine clinical practice. Recently, new standardized diagnostic methods to facilitate microbiome profiling has been published, such as using a dysbiosis index (DI) to characterize global changes of the microbiome in human patients with IBD and irritable bowel syndrome (IBS)[78]. This particular study found that the DI was able to differentiate the fecal microbiome of healthy individuals from patients with IBD or IBS. Our group has recently developed a fecal DI for dogs.[78] The fecal DI was tested in dogs with IBD and showed differential clustering of dogs with IBD compared to healthy control dogs.[79,80]

The canine fecal DI offers a broad-spectrum, reproducible, high throughput, easy to use method of quantifying the extent of dysbiosis that is especially suitable for clinical use[81]. Several studies investigated the feline intestinal microbiome using different

techniques.[74,82] However, there has not been a study investigating the intestinal microbiome of cats with confirmed IBD or alimentary lymphoma compared to healthy cats.

**1.3.5. Biomarkers and the metabolome**

Another approach to gain deeper understanding of the pathogenetic mechanisms in IBD or SCL is the characterization of the fecal metabolome. Metabolites are small molecules, with a low-molecular mass (usually < 1000 Da) that are formed in or are necessary for the metabolism[83,84]. Small molecules excreted in the feces are collectively known as the fecal metabolome. The metabolome comprises of molecules that have been metabolized by either the host or the microbiota. Untargeted metabolomic studies in people reported differences in metabolomic profiles between IBD patients and healthy controls, as well as between patients with different IBD subtypes.[85-87]

In contrast to the microbiome, the effect of medication on the metabolome profile appears to be negligible.[85,88,89] Our group has previously performed untargeted metabolomic profiling in healthy dogs and in dogs with acute diarrhea or IBD.[73,74] The serum metabolites 3-hydroxybutyrate, hexuronic acid, ribose, and gluconic acid lactone were significantly more abundant in dogs with IBD compared to healthy controls.[73] In dogs with acute diarrhea, serum concentrations of kynurenic acid and urine concentrations of 2-methyl-1H-indole and 5-methoxy-1H-indole-3-carbaldehyde were significantly decreased.[74] The potential discriminatory power as well as the robustness against confounding factors such as medication makes metabolomic profiling an attractive target for biomarker candidate identification.

15

Untargeted metabolomic studies in people with IBD have previously revealed several metabolites that are useful as diagnostic markers or even therapeutic targets. Specifically, bile acids (BA) were found to play a major role in people and dogs with IBD.[80,90] Primary BA are synthesized by hepatocytes but undergo metabolism by the microbiota during the passage through the intestinal tract to form secondary BA before they are eventually reabsorbed and undergo enterohepatic recirculation. In people with IBD, fecal secondary BA were found to be significantly decreased while sulphated forms of lithocholic acid (LCA) were increased during phases of active disease compared to phases of remission.[90] Another study of people with irritable bowel syndrome (IBS) showed discriminatory power for fecal bile acids. While the diarrheic form of IBS was associated with increased fecal concentrations of unconjugated BA cholic acid (CA), deoxycholic acid (DCA), and chenodeoxycholic acid (CDCA), the constipative form of IBS was associated with an increase of the secondary BA lithocholic acid (LCA).[90] Interestingly, as mentioned above, the bacterial pathobiont *B. wadsworthia* is a potential contributing factor to IBD. This bacterial species thrives in the presence of taurine-conjugated bile acids that appear to be abundant in the feces of patients with IBD and thus illustrates the tight interaction between the metabolome and the microbiome.[91] *In vivo* experiments supported the pro-inflammatory effect of these alterations to the mucosa, suggesting that interventions targeting BA may have therapeutic applications. Cholestyramine has been reported to be beneficial in patients with ulcerative colitis after proctocolectomy and ileoanal pouch anastomosis[92]. Similar to findings in people with IBD, our group recently reported significantly decreased secondary fecal BA in dogs

with IBD compared to healthy dogs[80]. Secondary fecal BAs normalized in most dogs with IBD that achieved remission when receiving corticosteroids[80].

To the author's knowledge there are no studies regarding the metabolomic profile in cats with IBD or SCL.

## 1.4. Hypotheses and objectives

The following hypotheses were formulated for this study:

i. Small intestinal biopsy specimens collected from a cohort of client-owned clinically healthy cats will show findings deemed to be "normal" based on WSAVA histopathology criteria , immunohistochemistry, and molecular clonality testing.

ii. Characterization of the mucosal proteome will identify biomarker candidates that may serve as clinically useful diagnostic markers for feline CE.

iii. Characterization of the fecal microbiome will identify biomarker candidates that may serve as clinically useful diagnostic markers for feline CE.

iv. Characterization of the fecal metabolome will identify biomarker candidates that may serve as clinically useful diagnostic markers for feline CE.

The objectives to prove or disprove the aforementioned hypotheses are:

17

1. Assessment of histopathology, immunohistochemistry, and molecular clonality testing of small intestinal biopsy specimens in clinically healthy client-owned cats.

    a. To assess the performance of tests currently considered the gold standard of diagnosis and differentiation of feline CE in a group of healthy client-owned pet cats.

    b. To verify the health status of this cohort of cats and use samples acquired from this group of cats for experiments mentioned under point 2 to 4.

2. Assessment of the intestinal proteome

    a. To describe the intestinal proteome of cats with IBD, SCL, and healthy control cats using two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) and liquid chromatography tandem-mass spectrometry (LC-MS/MS).

    b. To identify biomarker candidates with discriminatory power between those groups of cats.

3. Assessment of the fecal microbiome

   a. To describe the fecal microbiome of cats with IBD, SCL, and healthy control cats using high-throughput 16S rRNA Illumina Sequencing.

   b. To identify bacterial taxa associated with CE in cats.

4. Assessment of the fecal metabolome

   a. To describe the fecal metabolome of cats with IBD, SCL, and healthy control cats using Ultra-Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) coupled with High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS).

   b. To identify biomarker candidates with discriminatory power between those groups of cats.

# 2. RESULTS OF HISTOPATHOLOGY, IMMUNOHISTOCHEMISTRY AND MOLECULAR CLONALITY TESTING OF SMALL INTESTINAL BIOPSY SPECIMENS FROM CLINICALLY HEALTHY CLIENT-OWNED CATS[93]

Background: Histopathology, immunohistochemistry, and molecular clonality testing are metrics frequently used to diagnose chronic enteropathy (CE) in cats. However, normal values for these metrics have been based mainly on samples from cats that were relatively young, specific pathogen-free, or both.

Objectives: To describe results of histopathology, immunohistochemistry, and clonality testing of endoscopically derived biopsy specimens of the upper small intestinal tract from a cohort of clinically healthy client-owned cats.

Animals: Twenty clinically healthy client-owned cats $\geq 3$ years of age.

Methods: Tissue specimens were collected from the stomach and duodenum and evaluated single-blinded by a board-certified pathologist. In addition, samples were evaluated by routine immunohistochemistry and clonality testing. Cats were followed after the procedure for signs of CE.

Results: Integrated results from histopathology, immunohistochemistry, and clonality testing were interpreted as consistent with small cell lymphoma (SCL; n = 12), emerging SCL (n = 1), lymphocytic enteritis (n = 6), and pseudoclonality (n = 1). On

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[93]Reprinted with permission from "Results of Histopathology, Immunohistochemistry, and Molecular Clonality Testing of Small Intestinal Biopsy Specimens from Clinically Healthy Client-Owned Cats" by S Marsilio, MR Ackermann, JA Lidbury, JS Suchodolski, JM Steiner, 2019. *Journal of Veterinary Internal Medicine*, 33(2), 551-558, Copyright 2019 by Wiley.

follow-up, 3 cats eventually developed clinical signs of CE, of which 2 were euthanized 295 and 654 days post-endoscopy. The remaining 17 cats did not show clinical signs of CE after a median of 709 days (range, 219 – 869 days)

Conclusions and Clinical Importance: Intestinal biopsy specimens from clinically healthy client-owned cats commonly had abnormal findings on histopathology, immunohistochemistry, clonality testing or some combination of these without apparent clinical relevance. Current diagnostic metrics for diagnosing CE in cats may need modification to be applicable to the general population of cats.

## 2.1. Introduction

In 2008, the World Small Animal Veterinary Association (WSAVA) Gastrointestinal Standardization Group published histopathological standards for the diagnosis of gastrointestinal inflammation in endoscopic biopsy specimens from cats and dogs[25]. The histopathological standards include the description of normal and abnormal findings in the gastrointestinal tract of cats. Scoring sheets and pictorial templates of histopathological images were developed for the description and grading of morphological and inflammatory changes, thereby establishing a histopathologic standard of 'normal' and 'abnormal'[25]. However, most of the studies used for developing the WSAVA criteria were performed on full-thickness biopsy specimens from specific pathogen-free (SPF) colony cats that predominantly were relatively young, with most cats being between 5 and 18 months of age[28,29]. This reference group differs from the population to which the standard criteria are actually applied. More specifically, cats with chronic enteropathy (CE) usually are middle-aged to older, with a variety of

backgrounds and lifestyles[4,8,9,16,31,94]. Therefore, the question arises as to whether the standard criteria are applicable to the general population of cats presenting with possible CE, and whether the definition of 'normal' by WSAVA standards represents the findings in normal cats in the corresponding age group.

Immunohistochemistry and molecular clonality testing frequently are used as additions to routine histopathologic assessment, to differentiate inflammatory from neoplastic changes in cats with CE. Antigenic stimulation induces clonal expansion of memory lymphocytes as part of the physiologic immune response in inflammation.[95] This affects many different lymphocyte clones with a wide range of antigen receptor specificities and thus expansion and antigen receptor rearrangements become polyclonal[30]. Lymphocytic neoplasms comprise clones of a single or very few lymphocytic precursors, showing the same receptor rearrangement as their parent cell, resulting in monoclonal or oligoclonal receptor rearrangements[30]. Immunohistochemistry is used to determine whether a mixed population of T- and B-lymphocytes, indicative of inflammatory lesions, is present in the tissue or whether the lymphocytic population mainly consists of a single lymphocyte type, as seen in alimentary lymphomas in cats.

Clonality testing assesses the diversity of lymphocyte antigen receptor gene rearrangements, thereby differentiating clonal from polyclonal rearrangements. Polymerase chain reaction for antigen receptor rearrangements (PARR) is the most commonly used clonality assay for differentiating inflammatory bowel disease (IBD) from small cell lymphoma (SCL) in cats, because this test is widely available and can be

performed on formalin-fixed and paraffin-embedded (FFPE) tissue[34]. However, similar to the WSAVA histology criteria, the assay has been developed on tissue from SPF colony cats[34].

In this study, we characterize histopathological, immunohistochemical (for immunophenotyping), and clonality assay findings in endoscopically-derived biopsy specimens of the upper small intestinal tract from a group of clinically healthy, client-owned cats with demographic characteristics resembling those of the cats to which these tests are applied. We hypothesized that a cohort of client-owned clinically healthy cats would show findings deemed to be normal according to the current standard tests for CE.

## 2.2. Materials and methods

### 2.2.1. Cats

This prospective study was conducted at the Veterinary Medical Teaching Hospital, Texas A&M University. The study protocol was approved by the Texas A&M University Institutional Animal Care and Use Committee, and written owner consent was obtained for each cat before enrollment into the study. Twenty cats were included in the study.

### 2.2.2. Inclusion and exclusion criteria

Clinically healthy, adult, client-owned cats $\geq$ 3 years of age, undergoing an elective procedure requiring general anesthesia were eligible for enrollment in the study. Study eligibility initially was determined by an owner questionnaire on general and gastrointestinal health. The questionnaire covered the following areas: attitude, activity, appetite, drinking, urination, chronic illnesses, weight loss, vomiting, diarrhea, and

treatment with antibiotics, antacids, anti-inflammatory drugs, or corticosteroids. A physical examination was performed by a single board-certified internist (SM). Blood was collected from a peripheral vein or the jugular vein and the following tests were performed: CBC, serum biochemistry profile, serum total T4 concentration, and serum concentrations of cobalamin, folate, feline pancreatic lipase immunoreactivity (fPLI), and feline trypsin-like immunoreactivity (fTLI). Additionally, a serum feline immunodeficiency virus antibody test and feline leukemia virus antigen test were performed if the status of the cat was unknown (n = 4). Cats with gastrointestinal signs (e.g. weight loss, hyporexia, vomiting > twice per month, diarrhea) within 6 months before enrollment were excluded. In addition, cats with systemic diseases, chronic illnesses, or laboratory abnormalities that were deemed to be clinically relevant were excluded from the study. Cats with a serum cobalamin concentration < 350 ng/L also were ineligible. Finally, cats that had received any antibiotics, antacids, anti-inflammatory drugs, or corticosteroids within the past 6 months were excluded from the study.

### 2.2.3. Sample collection and processing

After a routine dental procedure under general anesthesia, all cats underwent gastroduodenoscopy. Six biopsy specimens each from the stomach and upper small intestinal tract (SI) were collected for histopathologic examination, immunohistochemistry, and clonality testing. In 1 cat anesthesia time and personnel allowed for an additional ileo-colonoscopy, whereas in 2 cats only duodenal biopsy specimens were collected because of a longer than usual set-up time.

Histopathologic examination of (H&E) hematoxylin and eosin-stained endoscopic FFPE tissue sections was performed by a single board-certified pathologist (MA) blinded to the clinical status of the cats (i.e., the pathologist was unaware that the samples were from healthy control cats). Findings were reported descriptively, and numerically scored according to the WSAVA histopathologic scoring system[24,25]. Briefly, morphological features (e.g. surface epithelial injury, crypt hyperplasia, crypt dilatation or distortion, and fibrosis or atrophy) and inflammatory changes (e.g. lamina propria lymphocytes, plasma cells, eosinophils, neutrophils, macrophages) were assessed histologically and assigned a score (normal = 0, mild = 1, moderate = 2, and marked = 3)[25].

Sections of FFPE tissue were sent to a single external laboratory for immunohistochemistry and clonality testing.*† Pathologists at the external laboratory were blinded to the health status of the cats. Biopsy sections were reassessed with H&E staining, and then immunohistochemistry and clonality testing were performed.

Immunohistochemistry was conducted using a stepwise approach. Staining for T-, B-, and natural killer- (NK) cell markers (CD3, CD79a, granzyme B, respectively) were performed at the external pathologist's discretion and based on the results of the H&E staining (i.e., size and distribution of mucosal lymphocytes).

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†The immunohistochemistry and molecular clonality analyses of biopsy specimens were performed at the Leukocyte Antigen Biology Laboratory, University of California Davis, SVM-PMI, One Shields Ave, 4206 VM3A, Davis, CA 95616 on a fee-for-service basis

Molecular clonality testing was conducted on at least 2 sections (each 25 µm) of FFPE tissue using PARR analysis. Total DNA content was measured before the PCR procedure to ensure that enough tissue was present for accurate PARR testing. Results from the H&E based histopathology, immunohistochemistry, and molecular clonality analysis were integrated and reported by the external pathologist.

**2.2.4. Follow-up**

All cats were followed up after endoscopy at various timepoints. An owner questionnaire on general and gastrointestinal health since endoscopy was used to assess the cat's health status. If abnormal findings were reported, owners were contacted and a more detailed history was obtained.

**2.3. Statistical analyses**

The association between the results of laboratory tests, histopathology, and clonality assays was assessed using chi-squared tests or a Fisher's exact test, as appropriate. Statistical significance was set at $P < .05$. Statistical analyses were performed using a statistical software package (GraphPad Prism, GraphPad Software, Inc, San Diego, California).

**2.4. Results**

**2.4.1. Study population**

Twenty cats were included in the study. Cats had a median age of 9.5 years (range; 3 – 18 years), median body weight of 5.0 kg (range; 2.6 – 10.8 kg) and median body condition score of 6 out of 9 (range; 5 – 9). There were 12 female spayed and 8

male neutered cats. Breeds included domestic shorthair (n = 12), domestic longhair (n = 3), Siamese (n = 2), Burmese (n = 1), Norwegian Forest Cat (n = 1), and Persian (n = 1).

According to the owner, 1 cat had had a short episode of acute, self-limiting diarrhea within the 6 months before the study. This cat had a minimally increased serum folate concentration (22.4 µg/L; reference interval, 9.7 - 21.6 µg/L). One cat had an increased fPLI concentration (15.6 µg/L; reference interval, ≤ 3.5 µg/L) without any current or prior associated clinical signs. Five cats had increased serum folate concentrations (25.3 µg/L, 27.3 µg/L, 33.8 µg/L, 62.5 µg/L and 65.5 µg/L) without any current or prior associated clinical signs.

Gastric biopsy specimens were available from 18 cats, and upper intestinal tract biopsy specimens were available from all 20 cats.

Demographic characteristics, histopathological findings, and results of molecular clonality testing are shown in Table 2.1. Detailed results for individual cats are shown in Appendix A, Table 6.1.

Table 2.1. Demographic data, histopathology, and molecular clonality testing

| number of cats | 20 |
|---|---|
| **demographic information** | |
| median age in years (range) | 9.5 (3 – 18) |
| median body weight in kg (range) | 5.0 (2.6 – 10.8) |
| median BCS (range) | 6 (5 – 9) |
| sex | 12 FS, 8 MN |
| breeds | 12 DSH, 3 DLH, 2 Siamese, 1 Burmese, 1 Norwegian Forest Cat, 1 Persian |
| **results for histopathology** | |
| median gastric WSAVA score (range) | 1.5 (0.5 – 3.5) |
| median duodenal WSAVA score (range) | 2.5 (1.5 – 5.5) |
| **results for molecular clonality assays on FFPE duodenal biopsy specimens (n = 20)** | |
| clonal rearrangements | 8 |
| clonal rearrangements in a polyclonal background | 5 |
| polyclonal rearrangements | 6 |
| pseudoclonal rearrangements | 1 |

Abbreviations: BCS, body condition score, 1-4: underweight, 5: ideal, 6-9 overweight; FS female spayed, FFPE formalin-fixed, paraffin-embedded, MN male neutered, DSH domestic shorthair, DLH domestic longhair

**2.4.2. Histopathologic results**

Sample number and quality were reported by the pathologist to be adequate for all cats. A detailed list of the WSAVA scores is shown in Appendix A. Table 6.1.

Histopathologic evaluation of H&E-stained gastric biopsy sections had abnormalities in all 18 available cats. Lymphocytic-plasmacytic gastritis was identified in all cats. This finding was reported to be minimal in 4, minimal to mild in 2, mild in 5, mild to moderate in 5, and moderate in 2 cats. One of the 2 cats with moderate lymphocytic-plasmacytic gastritis was reported to have focally extensive nodular lymphocytic and plasmacytic gastritis. Fibrosis was the most commonly reported morphologic abnormality, present in 17 cats (minimal in 3, minimal to mild in 2, mild in 11, moderate in 1); lymphocytic nodule formation was present in 2 cats and 1 cat had occasional mucosal cyst formation.

Histopathologic evaluation of duodenal biopsy specimens showed some degree of lymphocytic-plasmacytic mucosal infiltration in all 20 cats (minimal to mild in 4, mild in 4, mild to moderate in 6, and moderate in 4). In 2 cats, a diagnosis of SCL was made based on histopathology. In addition to a diffuse infiltration of the lamina propria with monomorphic small lymphocytes, both cats had moderate epithelial infiltration with small lymphocytes. Morphologic changes were present in 19 cats. The most common architectural change observed in the duodenum was crypt hyperplasia in 18 cats (minimal in 3, minimal to mild in 1, mild in 6, and mild to moderate in 7), followed by fibrosis in 4, and lacteal dilatation in 4. One cat was reported to have occasional crypt abscesses.

**2.4.3. Immunohistochemistry**

Lymphocytes infiltrating the lamina propria stained positive for CD3 in all cats. In 12 cats, a CD3+ epitheliotropic lymphocyte population was reported. In 5 cats, a mixed epitheliotropic and lamina proprial lymphocytic infiltrate staining positive for CD3 was identified. In 3 cases, the pathologist reported a CD3+ lymphocytic infiltrate without further comments on localization. In no case did the pathologist suggest that any additional stains were needed for a final diagnosis.

**2.4.4. PCR for antigen receptor rearrangements**

Molecular clonality testing of T cell receptor genes (TRG) was performed in all cats. In addition, clonality analysis using B-cell primers including IgH2, IgH3, and κ-deleting element (KDE) was performed on sections of duodenal biopsy specimens from 1 cat.

Molecular clonality testing of the TRG identified clonal rearrangements in duodenal biopsy specimens of 8 cats. In 5 cats, clonal rearrangements in a polyclonal background were reported. In 6 cats, including the single case in which T- and B-cell primers were used, rearrangements were determined to be polyclonal. In 1 cat, TRG clonality analysis was interpreted as pseudoclonal, likely because of insufficient DNA retrieval.

**2.4.5. Integrated interpretation based on H&E staining, immunohistochemistry, and PARR**

Results from H&E stains, immunohistochemistry, and PARR were integrated by the external pathologists and reported as case interpretations. Results were interpreted as consistent with duodenal SCL in 12 cats and emerging SCL in 1 cat. Six cats were

reported as having lymphocytic enteritis. In 1 cat the findings were deemed uninterpretable because of pseudoclonality.

## 2.4.6. Correlation among laboratory findings, histopathology, and results of clonality testing

No association was found between laboratory findings and histopathology or results of clonality testing (P = .28 and P = .18, respectively). Similarly, no association was identified between histopathology and results of clonality testing (P = .38).

## 2.4.7. Follow-up data

Follow-up data were available for all 20 cats. Two cats were euthanized because of signs of gastrointestinal disease, including weight loss and vomiting, 295 and 654 days post-endoscopy, respectively (Appendix A. Table 6.2, cases 10 and 19). Both cats were diagnosed previously with SCL based on the first histopathologic examination as well as laboratory results on H&E, immunohistochemistry, and PARR. The owner reported that the former cat had developed weight loss (approximately 1 kg) and frequent vomiting approximately 9 months after endoscopy. The cat underwent abdominal ultrasound examination before euthanasia during which thickened segments within the SI tract and abdominal lymphadenomegaly were identified. This cat did not receive any treatment. The second cat developed weight loss (1.3 kg), sarcopenia, vomiting, and chronic kidney disease (International Renal Interest Society stage 3, proteinuric, non-hypertensive). The cat was treated with prednisolone and budesonide before euthanasia eventually was elected. Neither cat was available for post-mortem examination.

Another cat developed severe non-self-limiting vomiting approximately 513 days post-endoscopy (Appendix A. Table 6.1, case 13). The owner reported that a CBC, serum biochemistry profile, and abdominal ultrasound examination had been within normal limits and that the cat's body weight had been unchanged after endoscopy. Upon treatment with a prescription hydrolyzed protein diet, signs of gastrointestinal disease ceased within days. At the time of study enrollment, this cat was the only 1 in which ileo-colonoscopy was performed in addition to gastro-duodenoscopy. Histopathology at that time showed mild diffuse and moderate nodular lymphocytic-plasmacytic gastritis with mild fibrosis, mild diffuse lymphocytic-plasmacytic duodenitis with minimal diffuse hyperplasia of crypts, minimal diffuse lymphocytic-plasmacytic ileitis, and mild diffuse lymphocytic-plasmacytic colitis with multifocal nodular lymphocytic aggregates. The WSAVA scores of the stomach, duodenum, and colon were 2.5, 2.0, and 2.0, respectively. Immunohistochemistry was consistent with a mildly epitheliotropic lymphocyte population staining positive for CD3 in both the upper and lower SI tract. Clonality testing identified polyclonal rearrangements in samples from the upper and lower SI tract, with small reproducible peaks within the polyclonal background, suggestive of a decreased T cell receptor repertoire. Lesions were interpreted as consistent with lymphocytic enteritis with mild epitheliotropism in the upper SI tract and lymphocytic enteritis within the lower SI tract.

Owners' responses to follow-up questionnaires for the remaining 17 cats indicated no signs of chronic gastrointestinal disease after a median of 709 days post-endoscopy (range, 219 – 869 days). Of these 17 cats, duodenal biopsy specimens previously had

been interpreted as consistent with SCL in 10, emerging SCL in 1, and lymphocytic enteritis in 5. One case was deemed uninterpretable due to pseudoclonality.

**2.5. Discussion**

To our knowledge, ours is the first study describing the results of histopathology, immunohistochemistry, and molecular clonality testing in endoscopically-obtained upper SI biopsy specimens of clinically healthy, client-owned cats with demographic characteristics similar to those of cats that present with signs of CE.

All 20 cats in our study had histopathological changes that are considered abnormal based on current WSAVA standards. Although many inflammatory changes were considered minimal to mild, most of the cats had inflammatory lesions that were rated as mild to moderate lymphocytic-plasmacytic enteritis. Two cats were diagnosed with SCL based on histopathology alone. Other histological features seen in our population of clinically healthy cats included lymphocytic nodule formation in the stomach, fibrosis, crypt hyperplasia, lacteal dilatation, and crypt abscesses.

Results of histopathologic studies of intestinal biopsy specimens from clinically healthy cats have been described before and are the basis for the WSAVA histopathological standards for the diagnosis of gastrointestinal inflammation in endoscopic biopsy specimens from both dogs and cats[24,25,28,29]. However, upon careful examination of the reference population for the WSAVA standards, the definition of a normal histological baseline becomes somewhat questionable. Although guidelines for establishing a normal histopathological baseline are lacking, guidelines exist for establishing reference intervals for laboratory values. Generally, a reference interval is

defined as "an interval that, when applied to the population serviced by the laboratory, correctly includes most of the subjects with characteristics similar to the reference group, but excludes others".[96] In other words, a reference should be established in a cohort of healthy individuals with otherwise the same characteristics as the patient population to which it is intended to be compared. The same principle likely should apply to establishing histopathologic standards. Depending on the study and underlying diagnosis of IBD or SCL, cats with CE have a reported median age between 6 and 13.5 years[4,8,9,16,31,94]. However, the WSAVA criteria were developed based mainly on full-thickness biopsies from SPF colony cats that were relatively young[28,29]. One study used adult cats of undetermined origin from an Animal Control Center in Japan[97]. However, the authors also stated that, based on the dental status, the cats were considered young adults. In addition, this study mostly contributed to the WSAVA criteria through the description of epithelial globular leukocytes, rather than normal architecture and total mucosal leukocytes.

The intestinal tract and its gut-associated lymphoid tissue (GALT) is the largest lymphoid organ in the body, with an extremely high plasticity and compensatory capacity[98]. With increasing age and chronic antigen exposure, gastrointestinal histology may change without necessarily representing a pathological condition. Therefore, most of the histopathologic lesions seen in our population of cats might in fact be normal for older cats.

On the other hand, clinical or subclinical CE is very common in geriatric cats, as well as in elderly people, with up to 40% of geriatric human patients reporting at least 1 gastrointestinal complaint during a routine physical examination[99]. Therefore, histopathological changes seen in our population also might reflect true subclinical disease. At least for the 2 cats diagnosed with SCL on histopathology, this appears likely, because those cats developed severe clinical signs of CE and eventually were euthanized approximately 10 and 22 months post-endoscopy. Another cat developed clinical signs of CE with severe vomiting approximately 17 months post-endoscopy. However, this cat had only minimal to mild histopathological changes in both the upper and lower SI tract, and the lymphocyte population was determined to be polyclonal on PARR. In addition, clinical signs subsided after treatment with a hypoallergenic diet. The long lag time between endoscopy and development of clinical signs, mild histopathological changes and polyclonality at the time of endoscopy, and response to diet imply that, in this particular cat, the clinical signs might have been caused by a new onset of CE unrelated to previous changes, and the disease might be categorized as food-responsive enteropathy. The remaining 17 of 20 cats had no clinical signs of chronic gastrointestinal disease after a median of 709 days post-endoscopy (range, 219 – 870 days), and thus subclinical disease appears less likely for this group of cats.

Finally, observer-dependent variability is another possible explanation for our results. Despite standardized scoring criteria, interpretation of histopathological findings may vary substantially among pathologists[26,27]. In addition, the extent of cellular infiltration in the lamina propria was judged subjectively, without using computer-

assisted morphometry methods. Therefore, observer-dependent subjectivity and error cannot be ruled out and another pathologist might have assessed and scored the biopsy specimens differently.

In addition to histopathological examinations by 1 pathologist, samples were sent to an external laboratory for immunohistochemistry and molecular clonality analysis of FFPE duodenal tissue samples. Pathologists at the external laboratory performed their own H&E-staining evaluation and integrated results from H&E-based histopathology, immunohistochemistry, and PARR to formulate a diagnosis and interpretation of each case as performed routinely on samples from cats submitted to the laboratory for diagnostic purposes. Clonality analysis of the TRG revealed clonal rearrangements in 13 of 20 cats with or without a polyclonal inflammatory background and thus they were interpreted as consistent with SCL (n = 12) or emerging SCL (n = 1). Only 6 cats were found to have polyclonal TCR rearrangements. One cat was found to have results consistent with pseudoclonality, most likely due to insufficient DNA retrieval. One possible explanation for the frequent finding of clonal rearrangements would be the presence of malignant but indolent lymphocyte clones. Again, this would appear to be a reasonable explanation for the 2 cats that were diagnosed with SCL on histopathology and much later developed clinical signs of CE. We cannot exclude the possibility that the remaining 11 cats had SCL elsewhere in the gastrointestinal tract with trafficking neoplastic lymphocytes causing positive clonality results. Lymphocyte trafficking is a well-known phenomenon, allowing naïve and memory T cells to be recruited to the lamina propria or epithelium of the intestinal tract by a process called lymphocyte

36

homing[100]. Trafficking of neoplastic (clonal) lymphocytes might cause detection of clonal TRG rearrangements in the present lymphocyte population without apparent histopathological changes. However, the long disease-free follow-up time for these cats makes this scenario somewhat unlikely.

Another explanation would be the occurrence of benign clones. In some instances, chronic antigenic stimulation may lead to disproportional proliferation of a lymphocytic subpopulation, resulting in true but benign clonal expansion, often in a polyclonal background[30]. Benign clonal expansion has been documented in humans[101,102] and dogs with infectious and autoimmune diseases, neoplasia, and drug-administration[103-105]. Other causes for the detection of clonality in the absence of neoplasia include canonical rearrangements of certain γδ T cell clones and non-specific amplification of sequences other than rearranged TRGs[30]. Benign clonal expansion is a plausible explanation, especially for the 5 cats in which clonality analysis identified clonal rearrangements in a polyclonal background. However, we did not find an association between extent of inflammation and results of the clonality assay. Also, even if such benign clonal expansion were to be the reason for the many cats that were positive for PARR, this would severely hamper the clinical usefulness of PARR.

The presence of pseudoclonal results may be another valid explanation for our findings. Pseudoclonal profiles may result from a lack of primers covering the rearranged genes, mutation of primer binding sites (common in somatic hypermutation in B-cells), absence of rearranged T cell receptor gamma chains (NK-cell neoplasms) or insufficient target DNA[30]. In PCR-based clonality assays, the amount of input DNA is

standardized and determined mainly by the size and amount of tissue available for DNA retrieval. However, the target DNA is the DNA that is amplified during the PCR (i.e. DNA from T cells in TRG clonality assays). With low numbers of lesional T cells, the ratio of target DNA to total DNA decreases and preferential amplification and pseudoclonality may occur despite adequate total DNA concentration and purity[30,48]. This is an important reason that clonality assays should always be interpreted in the context of histopathology and immunohistochemistry, and thus should be performed in the same laboratory, preferably by the same pathologist. In our study, 1 cat was reported to have pseudoclonal rearrangements, most likely because of low target DNA in the sample, and thus results were deemed uninterpretable. In all other cats, the clonality assays were judged to have sufficient input DNA for the assay to be performed and interpretations of integrated results from histopathology, immunohistochemistry, and PARR were reported for these cats. Polymerase chain reaction-based analysis of Ig/TCR rearrangements is widely used in human medicine and is considered to be the gold standard for clonality testing[48]. However, both false negative and false positive results have been a problem, especially in the early years of assay use. This has led to the formation of the EuroClonality (BIOMED-2) consortium and the development of standardized multiplex PCR assays for nearly all Ig/TCR targets in humans[48,106]. This standardization made it technically feasible to bring this test into a routine diagnostic setting. However, besides the analytical phase, pre- and post-analytical aspects should be considered. Thus, interpretation algorithms have been introduced that take into account peak heights and ratios to define truly clonal rearrangements[48,49,106-108]. Such

standardization is currently lacking between veterinary laboratories, and thus differences in primers, laboratory practices, and result interpretation among laboratories might explain our findings[49].

Since the introduction of clonality assays for the diagnosis of intestinal T cell lymphoma in cats in 2005, several studies have investigated the value of PCR-based clonality assays in the diagnosis of intestinal and extraintestinal T cell lymphoma in cats[31,34,109,110]. Subsequently, clonality assays have become the gold standard for the diagnosis and differentiation of lymphoma in cats[12]. However, similar to the WSAVA criteria, PARR for the molecular diagnosis of intestinal T cell lymphoma in cats was developed based on samples obtained from healthy, young (i.e., 12 – 18 months old) SPF colony cats and thus might not be representative of the target population. Although the sensitivity of PCR-based clonality assays performed on FFPE tissue generally is considered high (> 90%), a study in human patients with lymphoproliferative disease identified specificities as low as 54.3% in patients with reactive lesions, even with the use of standardized BIOMED-2 clonality assays[47]. A recent study in cats with CE reclassified cats diagnosed with IBD on the basis of histopathology as having SCL instead, based on their PARR analysis[7]. Our results, as well as results from human pathology, imply that reclassification based on clonality results alone may not be justified.

Our study had several limitations. So as not to severely prolong anesthesia time, endoscopy and collection of biopsy specimens were restricted to the upper SI tract and stomach in most of our cats. Thus, we cannot exclude the possibility that some cats had

SCL elsewhere inside or outside the intestinal tract, which might explain the number of clonal results in our study. However, once again, this seems unlikely because most of the cats never developed any clinical signs of CE even after having been followed for several month to years. In addition, we cannot entirely exclude the possibility of subclinical disease being present in this population of cats. Most of the cats in our study were slightly overweight with a median body condition score of 6 (range, 5 to 9 out of 9), and thus obesity could be viewed as a clinical abnormality. However, for a number of different diseases in humans, mild obesity has been shown to be associated with longer survival compared to patients with a body condition that is considered ideal[111]. Based on our clinical experience, most healthy geriatric cats are overweight, and restricting the body condition score to an ideal score of 5 out of 9 likely would have introduced a substantial bias into the study population. Our inclusion criteria permitted cats that were vomiting up to twice per month. In addition, some cats had laboratory abnormalities such as increased serum folate concentration or increased serum fPLI concentration without associated clinical signs. Cats that were vomiting either were long-haired cats vomiting predominately hairballs or had occasional vomiting up to twice per month without any other clinical signs. Both cats with SCL were among the 4 cats that had occasional vomiting, and thus we cannot exclude that this might have been an early sign of gastrointestinal disease. Progression of IBD to SCL over months to years has long been suspected, and inflammatory lesions frequently coexist with SCL[31,34]. Although results of histopathology and clonality testing did not correlate with clinical or laboratory abnormalities, subclinical CE remains a possible reason for the findings in our

study. Finally, because of to the stepwise approach the pathologists took for immunohistochemistry, tissue biopsy specimens did not routinely undergo staining for B- or NK-cells. Therefore, mixed infiltrates were likely missed in the tissue biopsy specimens. However, we intended not to interfere with the pathologists' approach and to assess whether this cohort of cats would be identified correctly as clinically healthy based on tests that currently are considered to be the gold standard for the diagnosis of CE in cats.

Conclusions

We characterized results of histopathology, immunohistochemistry, and molecular clonality testing in endoscopically-obtained upper SI biopsy specimens from healthy, client-owned cats with demographic characteristics resembling those of cats that present with signs of CE. Intestinal biopsy sapmples commonly had histopathologic findings considered abnormal based on current WSAVA standards. Similarly, results of clonality testing identified many cats with clonal rearrangements within this group of healthy cats. Our results suggest that histological scoring criteria may need to be revised and adapted to a more adequate reference population. Although the sensitivity of molecular clonality testing generally is considered to be high, our results imply that further assessment of the specificity of this diagnostic modality may be warranted.

# 3. CHARACTERIZATION OF THE INTESTINAL MUCOSAL PROTEOME IN CATS WITH INFLAMMATORY BOWEL DISEASE OR SMALL CELL ALIMENTARY LYMPHOMA

Feline chronic enteropathy (CE) is a common, spontaneously arising gastrointestinal disorder in cats and mainly comprises inflammatory bowel disease (IBD) and alimentary small cell lymphoma (SCL). Current diagnostic tests for diagnosis and differentiation of feline CE are expensive, invasive and lack specificity. Therefore, the identification of less invasive, more reliable biomarkers would be highly beneficial. We characterized the mucosal proteome in endoscopically obtained, small intestinal tissue biopsy specimens of 6 healthy control cats, 6 cats with IBD and 8 cats with SCL. The mucosal proteome was extracted by mechanical disruption and detergent-based cell lysis, differentially labeled and analyzed by 2-dimensional fluorescence difference gel electrophoresis and nanoflow liquid chromatography tandem mass spectrometry. A total of 2,349 spots were identified, of which 9 were differentially expressed with a $\geq$ 2-fold change between healthy cats and cats with IBD and SCL ($0.01 < P < 0.001$). Eight of these 9 spots were also differentially expressed between cats with IBD and cats with SCL (p $0.0015 < P < 0.04$). Nanoflow liquid chromatography tandem mass spectrometry identified proteins of the annexin and apolipoprotein families, and malate dehydrogenases in the differentially expressed spots.

Results of this study revealed differences between the mucosal proteome of healthy cats, cats with IBD, and cats with SCL. These proteins might hold potential for

the development of minimally-invasive biomarkers for the differentiation of IBD and SCL in cats with chronic enteropathy. Further studies to validate these findings are warranted.

## 3.1. Introduction

Feline chronic enteropathy (CE) is a spontaneously arising disorder in cats that is especially common in the senior cat population. It is defined as the presence of signs of gastrointestinal disease such as weight loss, vomiting, and diarrhea in the absence of infectious intestinal or extraintestinal causes for more than 3 weeks duration[112]. The most common causes of CE in cats are idiopathic inflammatory bowel disease (IBD) and alimentary small cell lymphoma (SCL)[1,7,12,31]. However, clinical signs are non-specific and diagnosis and differentiation require histopathological examination of tissue biopsies. Ambiguous cases frequently occur and require additional diagnostic measures such as immunohistochemistry and clonality testing [7,31,34]. Hence, current diagnostic modalities for feline CE are expensive and invasive. In addition, previous studies in humans revealed a specificity for clonality assays as low as 54.3% [47]. Although, the exact sensitivity and specificity of clonality assays in cats is currently unknown, a high false-positive rate appears to be of equal concern in feline medicine (unpublished data, Chapter 1 of thesis). Therefore, the identification of less invasive and more reliable biomarkers for the diagnosis and differentiation of feline CE would be highly desirable.

In 2001, the Biomarkers Definitions Working Group defined a biomarker as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic

intervention[50]. In inflammatory or cancerous conditions, biomarkers are commonly released by affected cells or in response to tissue dysfunction[113]. Biomarkers can be classified into proteins, small molecule metabolites, nucleotides and lipids[113]. The proteome describes the entire set of proteins of a cell, tissue or organism. Proteomics is the analysis of the proteome at a specific time point[114]. The proteome can be studied by a variety of different techniques including 2-dimensional fluorescence difference gel electrophoresis (2D DIGE)[115]. Discovery proteomics approaches have previously been utilized to identify unknown biomarkers for a variety of different diseases and conditions[60]. However, since protein biomarkers are commonly of low abundance and untargeted proteomics experiments are 'fishing expeditions', omics approaches commonly suffer from data noise that overwhelms the signal[60]. One approach to overcome this dilemma is to investigate the proteome in the affected tissue first, hypothesizing that the abundance of an unknown protein biomarker should be highest here[66]. Once a biomarker candidate has been identified, targeted approaches can be applied to other, less-invasively obtainable, samples such as biofluids and identify even low abundance proteins with a high sensitivity[66].

In this study we characterized the small intestinal mucosal proteome of cats with IBD and SCL by an untargeted proteomics approach using 2D DIGE and nanoflow liquid chromatography tandem mass spectrometry. Results were compared to those of a group of healthy control cats, with similar demographic characteristics. We hypothesized that the proteome would differ between these three groups and that 2D DIGE was able to identify biomarker candidates with the potential to differentiate IBD from SCL in cats.

**3.2. Materials and methods**

**3.2.1. Study approval and enrollment.**

This prospective study was conducted at the Veterinary Medical Teaching Hospital, Texas A&M University. The study protocols were approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC 2014-0369 CA and IACUC 2015-0276 CA). All experiments were performed in accordance with relevant guidelines and regulations. Cat owners provided written informed consent prior study enrollment.

**3.2.2. Patients and procedures**

Fourteen cats with clinical signs of chronic enteropathy (6 IBD cats and 8 SCL cats) and 6 control cats were recruited from the hospital population at the Small Animal Hospital at Texas A&M University, College Station, Texas, or the Veterinary Specialty Hospital, San Diego, California. Cats with clinical signs of chronic enteropathy (i.e., weight loss, hyporexia, vomiting, diarrhea) of ≥ 3 weeks duration were eligible for enrollment. Cats that had received corticosteroids within 4 weeks prior to fecal sampling were excluded from the study. All cats in this group underwent gastro-duodenoscopy and ileo-colonoscopy for diagnostic purposes. Histopathologic examination of H&E stained endoscopic formalin-fixed, paraffin-embedded (FFPE) tissue sections was performed by a single board-certified pathologist blinded to the clinical status of the cats. In addition, samples underwent immunohistochemistry and PCR for antigen receptor rearrangements (PARR). A final diagnosis of IBD (n=6) or SCL (n=8) was

reached upon integration of results from histopathology, immunohistochemistry, and PARR.

In addition to diagnostic biopsies, six biopsies were taken from the upper small intestinal tract, collected individually into pre-chilled tubes, and immediately snap-frozen in a methanol-dry ice bath. Samples taken at the Veterinary Specialty Hospital, San Diego were kept on dry ice and shipped to the Texas A&M Gastrointestinal Laboratory and immediately stored at -80°C upon arrival. Samples collected at the Veterinary Medical Teaching Hospital were transferred to the Texas A&M Gastrointestinal Laboratory and immediately stored at -80°C until 2D DIGE analysis.

Clinically healthy, adult, client-owned cats ≥ 3 years of age, undergoing an elective procedure requiring general anesthesia were eligible for enrollment into the study. Cats were deemed healthy based on a client-questionnaire, physical examination, and laboratory testing including complete blood count, serum biochemistry profile, serum total T4, and serum concentrations of cobalamin, folate, feline pancreatic lipase immunoreactivity (fPLI), and feline trypsin-like immunoreactivity (fTLI). Cats with gastrointestinal signs (i.e., weight loss, hyporexia, vomiting > 2x/ month, or diarrhea) within 6 months prior to enrollment were excluded. Cats that had received any antibiotics, antacids, anti-inflammatory drugs, or corticosteroids within the past 6 months were excluded from the study. Following a routine dental procedure under general anesthesia, all cats underwent gastroduodenoscopy. Six biopsy specimens each from upper small intestinal tract were collected for histopathologic examination, immunohistochemistry, and clonality testing. An additional six biopsies were collected

from the small intestinal tract as described above and immediately stored at -80°C until 2D DIGE analysis.

### 3.2.3. Protein processing and labeling.

Intestinal biopsy samples from each case were pooled but cases were extracted separately. Biopsy specimens were transferred into a 0.2ml ground glass pestle and tube tissue grinder and homogenized in 10 mM Tris-HCl/1% CHAPS (Pierce, Biotechnology, Waltham, MA) buffer containing DNAse (Sigma Chemical) and protease inhibitors (Roche). The proteome from the intestinal biopsies of each case was precipitated with chloroform/methanol[116] and dissolved in 100 μl of DIGE labeling buffer (30 mM Tris, 7M urea, 2M thiourea, 4% CHAPS, pH 8.5 buffer). The protein concentration of each protein extract was determined by the Bradford protein assay using bovine serum albumin as a standard[117].

### 3.2.4. 2-Dimensional fluorescence difference gel electrophoresis

Protein extracts were fluorescently labeled by reacting 50 μg of protein with 200 pmol Cy 2 or Cy 5 (CyDye DIGE Fluors, GE Healthcare). In order to eliminate gel-to-gel variation and facilitate comparison, a pooled internal standard, containing equal parts of all samples was additionally prepared. The internal standard was labeled with Cy3. The labeling reaction was conducted for 30 min at 4°C in the dark and subsequently quenched by the addition of 1 μl of 10 mM lysine (Sigma) for 10 min.

All protein extracts were separated by 2D DIGE[115] within the same run using a total of 10 analytical gels. Each gel was loaded with two samples each from a different group (i.e., healthy, IBD, or SCL). In addition, all gels contained equal portions of the

pooled internal standard. Samples for each gel were combined and rehydration buffer

(7M urea, 2M thiourea, 4% CHAPS, 0.5% Pharmalyte (GE Healthcare, Uppsala,

Sweden), 40 mM dithiothreitol (DTT), and 0.002% bromophenol blue) was added to a

final volume of 450 μl. Pooled samples were used to rehydrate an immobilized pH

gradient strip (24 cm; pH 3–10NL; GE Healthcare, Uppsala, Sweden) by passive

diffusion overnight at room temperature. Isoelectric focusing was performed on an IPG

Phor 2 horizontal electrophoresis system (GE Healthcare, Uppsala, Sweden) with a

program of 0.5 kV for 1h, ramping to 1 kV over 1 h, ramping to 8 kV over 2.5 h, holding

at 8 kV until 110 kV*h, and holding at 0.5 kV for 1.5 h.  Each focused strip was then

equilibrated in two steps: 15 minutes in a reducing equilibration buffer (6Murea, 50 mM

Tris-HCl, pH 8.8 with 30% (v/v) glycerol, 2% (w/v) SDS, 0.01 bromophenol, and 10

mg/mL DTT), followed by 15 minutes in an alkylating equilibration buffer where DTT

was replaced by 25 mg/mL iodoacetamide. The equilibrated IPG strips were then placed

directly on top of polymerized 12% SDS gels and sealed with an agarose sealing

solution (25 mM Tris, 192 mM, glycine, 0.1% SDS, 0.5% (w/v) agarose, and 0.02%

bromophenol blue). Gels were run in cooled tanks on an Ettan Dalt-12 electrophoresis

system (GE Healthcare) at 1W per gel until the bromophenol blue dye front reached the

bottom of the gel.

### 3.2.5. Image acquisition and analysis.

Gel image acquisition was performed at an excitation wavelength of 473 nm for

Cy2, 532 nm for Cy3, and 635 nm for Cy5 labeled samples using a Typhoon FLA 9500

laser scanner (GE Healthcare) at 100 μm resolution. Following scanning, all gels were fixed overnight in 10% methanol and 7.5% acetic acid until spot picking.

Gel images were processed using DeCyder 2-D Differential Analysis Software (v 6.5, GE Healthcare). The software uses various algorithms specifically designed for multiplexed 2D image experiments. Image analysis consist of the following steps: spot detection, background subtraction, in-gel normalization, gel artifact removal, gel-to-gel-spot matching, and statistical analysis. Spot detection was verified visually. Each spot consists of pixels of different intensities which describe how bright the pixel is and translates into a numeric value for each pixel. Thus, each spot has an intensity value that is calculated as the average pixel intensity for the spot area. The Biological Variation Analysis module was used for normalization of spot intensities. To account for gel-to-gel variability the internal standard included in each gel served as the reference point for normalization. Normalization for the spot intensities was performed by comparing individual intensities against the standard value and generating a fold change referred to the standard.

Gel images were matched and protein expression levels between different groups were compared using one-way ANOVA and multiple comparison tests included in the statistical software package. Statistical significance was set at $p < 0.05$. Proteins with an absolute fold change value > 2.0 between groups were selected for further analysis.

### 3.2.6. Spot picking and protein processing.

Ettan Spot Handling Workstations (GE Healthcare, Chicago, IL USA) were used to cut out the selected protein spots from the protein gel, perform an in-gel tryptic

digestion with recombinant porcine trypsin (Promega, Madison, WI) and extract the peptides from the gel. Extracted trypsin peptides were concentrated by SpeedVac. Protein identification was achieved by nanoflow liquid chromatography tandem mass spectrometry. Subsequently, peptides from the MS were identified using the MASCOT search engine. The MASCOT program (v2.2) searched the mouse genome using the following parameters for protein identification: 1) one missed cleavage by trypsin; 2) monoisotopic peptide masses; 3) peptide mass tolerance of 1.2 Da; and 4) fragment mass tolerance of 0.8 Da. Further, oxidation of methionine (variable modification) and carbamidomethylation (fixed modification) of cysteine were taken into consideration by MASCOT in the protein identification. Peptides were matched to proteins at a minimum of two peptides. Protein identification was verified by Scaffold (Proteome Software, Portland, OR).

### 3.2.7. Statistical analyses

Patient demographics were compared by the Mann-Whitney or Fisher's exact test. Differences in protein abundance between groups were analyzed by one-way ANOVA. *Post-hoc* testing was performed using student's t test. Significance was set at $p < 0.05$.

### 3.3. Results

Patient demographics are shown in Table 3.1. Cats with SCL were significantly older that cats with IBD or healthy controls. In addition, cats with IBD or SCL had a significantly lower body condition score. There was no difference for the distribution of sexes between groups.

A total of 2349 matching spots were identified of which 9 were differentially expressed between healthy controls, IBD cats, and SCL cats (0.01 < P < 0.001; Table 3.2). Eight of these 9 were also differentially expressed between cats with IBD and cats with SCL (0.0015 < P < 0.04). Spots 1063 and 1065 were immediately adjacent to each other and showed a 3.6-fold and 3.2-fold increased protein abundance, respectively. Cytosolic and mitochondrial malate dehydrogenases (MDH 1 and 2), Na(+)/H(+) exchange regulatory cofactor NHE-RF1, and inorganic pyrophosphatase (PPi) were identified by tandem mass spectrometry in both spots. Spot 1112 showed a 5.6-fold increase between healthy cats and cats with SCL and a 3.8-fold increase between cats with IBD and those with SCL. This spot contained annexin 5, ADP-sugar pyrophosphatase isoform X1, an adiponectin precursor, and phosphoglycolate phosphatase. The sole protein identified in spot 1116 was Annexin A4 (ANXA4) with an increased abundance in cats with SCL of 2.3-fold and 2-fold compared to healthy cats and cats with IBD, respectively. Spot 1301 was the only one of the 9 protein spots that showed a decreased abundance when comparing cats with SCL or IBD to healthy cats (-2.1 and - 2.4-fold, respectively). This spot contained Apolipoprotein A-1. A complete list of spot numbers, identified proteins and fold-changes is shown in Table 3.2.

Table 3.1. Demographic characteristics of healthy cats, cats with idiopathic inflammatory bowel disease (IBD) and cats with alimentary small cell lymphoma (SCL).

| | Healthy | IBD | SCL | P value | Healthy vs IBD | Healthy vs. SCL | IBD vs. SCL |
|---|---|---|---|---|---|---|---|
| **Number of cats** | 6 | 6 | 8 | NA | NA | NA | NA |
| **Median age in years (range)** | 8 (3 - 10) | 6.5 (2 - 10 | 12 (7 - 14) | 0.003ᵃ | >0.999 | 0.113 | 0.008 |
| **Median BW in kg (range)** | 5.04 (4.01 – 6.47) | 4.61 (2.49 – 4.99) | 4.12 (2.96 – 5.24) | 0.194ᵃ | NA | NA | NA |
| **Median BCS (range)** | 5 (5-9) | 4 (3-5) | 3.5 (2-6) | 0.020ᵃ | 0.053 | 0.057 | >0.999 |
| **Sex** | 2 FS, 4 MN | 2 FS, 4 MN | 3FS, 5 MN | 0.037ᵇ | NA | NA | NA |
| **Breeds** | 6 DSH | 1 DMH, 2 DLH, 3 DSH | 7 DSH, 1 DLH | NA | NA | NA | NA |

BCS, body condition score: 1-3: underweight, 4-5 ideal, 6-9 overweight[118]. ᵃKruskal-Wallis test, ᵇ Chi-square test, ᶜ Dunn's *post hoc* test

Table 3.2. Summary of proteins identified from differentially expressed spots.

| Spot | Identified Protein | GO Biological process | Fold $\nabla^a$ | Fold $\nabla^b$ | Fold $\nabla^c$ | # Peptides | P value |
|---|---|---|---|---|---|---|---|
| 1063 | Inorganic pyrophosphatase | Metabolic processes, negative prognostic indicator for certain cancer types | ns | 4.2 | 3.64 | 8 | 0.0014 |
| 1063 | Na(+)/H(+) exchange regulatory cofactor NHE-RF1 | Cytoskeleton, part of Wnt pathway | ns | 4.2 | 3.64 | 10 | 0.0014 |
| 1063 | Malate dehydrogenase, cytoplasmic | Metabolic processes, supports glycolysis in cancer cells | ns | 4.2 | 3.64 | 3 | 0.0014 |
| 1063 | Malate dehydrogenase, mitochondrial | Metabolic processes | ns | 4.2 | 3.64 | 2 | 0.0014 |
| 1065 | Inorganic pyrophosphatase | Metabolic processes, negative prognostic indicator for certain cancer types | ns | 3.55 | 3.21 | 7 | 0.0018 |
| 1065 | Na(+)/H(+) exchange regulatory cofactor NHE-RF1 | Cytoskeleton, part of Wnt pathway | ns | 3.55 | 3.21 | 8 | 0.0018 |
| 1065 | Malate dehydrogenase, cytoplasmic | Metabolic processes, supports glycolysis in cancer cells | ns | 3.55 | 3.21 | 4 | 0.0018 |
| 1065 | Malate dehydrogenase, mitochondrial | Metabolic processes | ns | 3.55 | 3.21 | 2 | 0.0018 |
| 1112 | Annexin A5 | Negative regulation of apoptotic processes | ns | 5.59 | 3.84 | 17 | 0.0045 |
| 1112 | ADP-sugar pyrophosphatase isoform X1 | Energy metabolism | ns | 5.59 | 3.84 | 3 | 0.0045 |
| 1112 | Adiponectin precursor | Adiponectin: control of lipid metabolism and insulin-sensitivity, anti-inflammatory | ns | 5.59 | 3.84 | 3 | 0.0045 |
| 1112 | Phosphoglycolate phosphatase | Metabolic processes | ns | 5.59 | 3.84 | 2 | 0.0045 |
| 1116 | Annexin A4 | Membrane-fusion, Exocytosis, de-regulated in a variety of cancers | ns | 2.25 | 1.99 | 18 | 0.0063 |
| 1202 | Rho GDP-dissociation inhibitor 1 | Control Rho proteins involved in cell polarity, migration and division, associated with metastasis and resistance to drug-induced apoptosis | ns | 3.25 | 3.58 | 7 | 0.0120 |
| 1202 | Endoplasmic reticulum resident protein 29 | Protein processing within the endoplasmic reticulum | ns | 3.25 | 3.58 | 4 | 0.0120 |

[a]Fold change IBD vs healthy P = 0.004; [b]Fold change SCL vs healthy 0.001 < P < 0.02;
[c]Fold change SCL vs IBD 0.0015 < P < 0.04; ns, not significant

Table 3.2. Continued. Summary of proteins identified from differentially expressed spots.

| Spot | Identified Protein | GO Biological process | Fold $\nabla^a$ | Fold $\nabla^b$ | Fold $\nabla^c$ | # Peptides | P value |
|---|---|---|---|---|---|---|---|
| 1216 | Rho GDP-dissociation inhibitor 1 | Control Rho proteins involved in cell polarity, migration and division, associated with metastasis and resistance to drug-induced apoptosis | ns | 6.3 | 5.48 | 12 | 0.0005 |
| 1216 | Rho GDP-dissociation inhibitor 2 | Cytoskeleton | ns | 6.3 | 5.48 | 5 | 0.0005 |
| 1216 | Endoplasmic reticulum resident protein 29 | Protein processing within the endoplasmic reticulum | ns | 6.3 | 5.48 | 7 | 0.0005 |
| 1301 | Apolipoprotein A-I | HDL assembly and reverse transport of cholesterol to the liver, protective role in cancer development | -2.08 | -2.4 | ns | 5 | 0.0029 |
| 1575 | Nucleoside diphosphate kinase B | Metabolic processes | ns | 2.56 | 2.66 | 4 | 0.0039 |
| 1577 | Nucleoside diphosphate kinase B | Metabolic processes | ns | 2.72 | 2.63 | 5 | 0.0005 |

[a]Fold change IBD vs healthy $P = 0.004$; [b]Fold change SCL vs healthy $0.001 < P < 0.02$;
[c]Fold change SCL vs IBD $0.0015 < P < 0.04$; ns, not significant

**3.4. Discussion**

To our knowledge, this is the first study characterizing the mucosal proteome in clinically healthy, client owned cats, cats with IBD, and cats with SCL. This 2D DIGE based approach revealed 9 differentially expressed protein spots between the three groups, of which 8 were also differentially expressed in the small intestinal mucosa of cats with IBD compared to cats with SCL with a minimum fold change of 200% (absolute 2.0-fold change). Within these spots, 14 proteins warranting further investigations as potential biomarkers for feline CE were identified with mass spectrometry (Table 3.2).

Several of these proteins have previously been found to be involved in inflammatory or neoplastic processes, while others do not appear to have an immediate connection with a pathologic condition. However, the same protein may have a variety of different biological or pathophysiological functions dependent location, time of translation from RNA, post-translational modifications or accumulation in tissue. In addition, many biological functions are still to be elucidated. Therefore, the lack of an apparent inflammatory or cancer-related pathway does not necessary exclude a protein's value as a potential biomarker.

Inorganic pyrophosphatase (PPi) is a ubiquitous, well conserved protein involved in energy metabolism DNA replication, protein synthesis, and calcium release from mitochondrial stores[119]. Dysregulated cellular PPi production, degradation, and transport have been associated with various disease states[119]. For instance, increased PPi levels were previously found in plasma from humans with osteoarthritis, pseudogout,

acromegaly, and uremia[120]. In addition, overexpression of cytosolic PPi has been described in different types of cancer in humans, such as breast cancer[121-123], lung cancer[124], ovarian cancer[125], hepatocellular carcinoma[126], and colorectal cancer[127]. Cytosolic PPi has also been shown to be associated with cell migration, invasion[128], and proliferation of neoplastic cells[129] *in vitro*. Besides its potential as a disease biomarker, PPi may also be of prognostic significance as it has been shown to correlate to poor outcomes in humans with ovarian cancer[130] and gastric cancer[131] in people. Based on protein spot abundance, we found PPi to be increased in cats with SCL compared to healthy cats and to cats with IBD. In the light of previous findings in humans and animal models, PPi might similarly have diagnostic and/or prognostic implications in cats with CE.

Another protein of interest identified in our study was Annexin A4 (ANXA4). ANXA4 is a calcium/phospholipid-binding protein involved in membrane fusion and exocytosis. Dysregulations of ANXA4 have been described in a variety of different cancer types in humans[132]. Overexpression of ANXA4 is also associated with tumor progression and chemotherapy resistance in a multitude of tumors, including gastrointestinal neoplasia such as esophageal, gastric, colorectal, pancreatic and hepatic cancer[132]. This study identified ANX4 as the sole feline protein in spot 1116. This spot displayed a 2.25 fold increase in cats with SCL compared to healthy cats and a 2 fold increase compared to cats with IBD.

Malate dehydrogenases (MDH) catalyze the NAD/NADH-dependent interconversion of malate and oxaloacetate, which is part of crucial metabolic pathways including the citric acid pathway[133]. At least two isoforms, cytosolic (MDH 1) and mitochondrial (MDH 2), can be differentiated based on their subcellular location and their specificity for the coenzyme NAD or NADP[133]. Interestingly, MDH 1 and 2 share between 93.7 and 98.8% sequence homology with other mammalian species[134]. However, sequence homology between feline MDH 1 and 2 is only 16.8%[134], making these two proteins very distinguishable. MDH 1 and 2 have been found to facilitate glycolysis in proliferating cells and cancer *in vitro* including a canine lymphoma cell line[135-137]. Our study revealed MDH 1 and 2 to be present in two adjacent spots with a 3.2 to 4.2 fold increased abundance in cats with SCL compared to healthy cats and cats with IBD.

Other proteins found to be increased in the intestinal mucosa of cats with CE compared to healthy cats included Annexin A5 (an apoptotic marker), Na(+)/H(+) exchange regulatory cofactor NHE-RF1 (a scaffold protein potentially enhancing the Wnt pathway), and endoplasmic reticulum protein 29 (endoplasmic reticulum secretion factor, which is upregulated in various cancers[138]).

The only protein that revealed to have a decreased abundance based on protein spot intensity was Apolipoprotein A1 (Apo A1). Apo A1 is synthesized in the liver and small intestine and subsequently released into the bloodstream where it, in concert with other lipids, forms high density lipoprotein (HDL) particles[139]. HDL particles reversely transport cholesterol from tissue back to the liver where it is taken up for excretion or

further processing[139]. HDL is known for its protective role against cardiovascular disease. However, recently Apo A1 and HDL have been in the focus of cancer research after epidemiological studies showed an inverse relationship between HDL and cancer risk[140]. Due to a high tumor cell proliferation rate, cancer cells generally have a high demand for cholesterol[141]. Several studies found an inverse correlation between HDL-cholesterol and risk of a large variety of different cancers, risk of relapse and general treatment outcome[139,142]. Conversely, increasing levels of Apo A1, due to upregulated gene expression or treatment with Apo A1, have been found to be protective of tumor growth and even exerts anti-tumor activity in mouse models[143]. These studies imply a causal inverse relationship between Apo A1 and cancer development. In this study Apo A1 was identified as the sole protein in spot 1301 with a -2.1 and -2.8 fold decrease in cats with IBD and cats with SCL compared to healthy cats, respectively. No significant differential expression was found between IBD and SCL. Further studies to investigate the role of Apo A1 as a diagnostic and prognostic marker for cats with CE are warranted.

This study has several limitations. Several proteins were identified by nanoflow liquid chromatography tandem mass spectrometry in more than one gel spot. However, these spots were mostly adjacent to each other within the same molecular weight range but different first-dimension, isoelectric focusing separation. These findings are mostly likely due to post-translational modifications of the same protein. Post-translational modifications such as phosphorylation, glycosylation, methylation, and S-nitrosylation often have minimal impact on the molecular weight of a protein, but can shift its isoelectric point by several pH units[144].

In addition, a small sample size is a limiting factor in many omics experiments in general and in in gel-based experiments in particular. Our gel system allowed us for a maximum of 12 gels to be run in parallel. In order to reduce technical variability, samples should be run within the same experiment at the same time limiting the number of samples that can be used.

In this clinical study, we used client-owned cats that either presented for signs of chronic enteropathy (i.e., IBD and SCL groups) or as a part of a regular health check (i.e., healthy control cats). Therefore, factors such as genetics and environment were not controlled and likely increased the biological variability. To reduce biological variability and thus data noise as much as possible, we included only cats that were categorized as healthy or as having IBD or SCL based on all tests that are available today (i.e., histopathology, immunohistochemistry and clonality assays). Only cases that could be definitively classified as either IBD or SCL were included, while cases that showed inconsistent results between tests were excluded from the study. However, matching groups for all other factors would have made this study less clinically relevant.

In conclusion, this 2D DIGE based proteomics study found several proteins differentially expressed in the small intestinal mucosa of cats with IBD or SCL compared to healthy control cats. Among these proteins of increased abundance were proteins of the annexin family, PPi and MDH 1 and 2. The only protein found to be decreased in cats with either IBD or SCL was Apo A1. Out of 9 protein spots found to de differentially expressed between groups, 8 were also differentially expressed between IBD and SCL. Proteins in these spots might be suitable as biomarkers to differentiate

IBD from SCL. Further studies to verify these findings with a quantitative or semi-quantitative method and eventually measure these proteins in samples that can be acquired with less invasive procedures, such as serum or urine, are warranted.

# 4. CHARACTERIZATION OF THE FECAL MICROBIOME IN CATS WITH INFLAMMATORY BOWEL DISEASE OR ALIMENTARY LYMPHOMA

Feline chronic enteropathy (CE) is a common gastrointestinal disorder in cats and mainly comprises inflammatory bowel disease (IBD) and small cell lymphoma (SCL). Both IBD and SCL in cats share features with chronic enteropathies such as IBD and monomorphic epitheliotropic intestinal T-cell lymphoma in humans. The aim of this study was to characterize the fecal microbiome of 38 healthy cats and 27 cats with CE (13 cats with IBD and 14 cats with SCL). Alpha diversity indices were significantly decreased in cats with CE (OTU p = 0.003, Shannon Index p = 0.008, Phylogenetic Diversity p = 0.019). ANOSIM showed a significant difference in bacterial communities, albeit with a small effect size ($P = 0.023$, R = 0.073). Univariate analysis and LEfSE showed a lower abundance of facultative anaerobic taxa of the phyla *Firmicutes* (families Ruminococcaceae and *Turicibacteraceae*), *Actinobacteria* (genus *Bifidobacterium*) and *Bacteroidetes* (i.a. *Bacteroides plebeius*) in cats with CE. The facultative anaerobic taxa *Enterobacteriaceae* and *Streptococcaceae* were increased in cats with CE. No significant difference between the microbiome of cats with IBD and those with SCL was found. Cats with CE showed patterns of dysbiosis similar to those in found people with IBD.

**4.1. Introduction**

Feline chronic enteropathy (CE) is common in elderly cats and is defined as the presence of clinical signs of gastrointestinal disease for more than 3 weeks in the absence of infectious intestinal diseases (e.g., parasites) and extraintestinal causes (e.g., renal disease, hyperthyroidism)[10].

Feline CE mainly comprises inflammatory bowel disease (IBD) and small cell lymphoma (SCL)[1,7,31].

Diagnosis and differentiation can be challenging as clinical signs might be virtually the same in both disease entities[1,19]. Today, the diagnosis of IBD or SCL is based on histopathologic examination of tissue biopsies acquired under general anesthesia[24,25]. Hence, the diagnosis of IBD and SCL is elaborate, expensive, time- and resource consuming and invasive. Treatment of IBD and SCL is usually based on immunosuppression using various steroids and cytotoxic medication such as chlorambucil or cyclosporine[8]. Therefore, less invasive diagnostic and treatment modalities would be highly desirable.

The intestinal microbiome plays a substantial role in modulating the host's immune system within and beyond the gastrointestinal tract. Studies in people and dogs with IBD have found alterations in the composition of the intestinal microbiome that might impair the host's health status[145-147]. These changes are commonly referred to as dysbiosis[147]. Despite variations among studies, species, and individuals, common themes characterize intestinal dysbiosis. Across different species, three main hallmarks of dysbiosis have been described: a reduction in overall bacterial diversity (alpha

diversity)[71,73,148,149], a decreased stability of microbial communities and thus a higher fluctuation rate over time[150], and a reduction in obligately anaerobic taxa of the phyla *Firmicutes* and *Bacteroidetes* at the expense of an increase in facultative anaerobes, including members of the family *Enterobacteriaceae*[71-73,148,150-153]. Dysbiosis has been described in people with various forms of enteropathy, including ulcerative colitis, Crohn's disease, and colorectal cancer, and it might be a driver or consequence of chronic inflammation and malignant transformation[154]. Feline IBD and SCL share some features with chronic enteropathies in people. Both human and feline IBD is characterized by chronic inflammatory changes in the gastrointestinal tract[7,155]. Feline SCL is characterized by a monomorphic infiltration of the intestinal mucosa with small to medium lymphocytes, mostly in the small intestine and is often associated with epitheliotropism[31,38]. This histologic appearance resembles that of monomorphic epitheliotropic intestinal T-cell lymphoma (MEITL) in people, formerly known as enteropathy associated T-cell lymphoma Type 2 (EATL Type 2)[38,43]. Whereas SCL is quite common in cats, MEITL is rare in people, and thus large intersectional studies are scarce[38]. Both IBD and SCL occur spontaneously and frequently in cats, and thus the cat could be an interesting model for IBD or MEITL in people.

Several studies have characterized the fecal microbiome in cats with acute and chronic diarrhea[71,156]. However, in previous studies the cats were categorized based on clinical signs without confirmation of the underlying disease process. This study aimed to characterize and compare the fecal microbiome in healthy cats and cats with histopathologically confirmed CE (IBD or SCL).

## 4.2. Materials and methods

### 4.2.1. Animals

This prospective study was conducted at the Veterinary Medical Teaching Hospital, Texas A&M University. The study protocol was approved by the Texas A&M University Institutional Animal Care and Use Committee and all methods were performed in accordance with relevant guidelines and regulations.

The health status of cats in the group considered healthy was verified by an owner questionnaire on general and gastrointestinal health. The questionnaire covered the following areas: attitude/activity, appetite, drinking, urination, chronic illnesses, weight loss, vomiting, diarrhea, and treatment with antibiotics, antacids, anti-inflammatory drugs, or steroids. In 22 cats, physical examination was performed by a single board certified internist (SM). Blood was collected from a peripheral vein or the jugular vein and the following tests were performed: complete blood count, serum chemistry profile, total T4, cobalamin, folate, feline pancreatic lipase immunoreactivity (fPLI), and feline trypsin-like immunoreactivity (fTLI). Cats with gastrointestinal signs (weight loss, hyporexia, vomiting > 2x/ month, diarrhea) within 6 months prior to enrollment were excluded. In addition, cats with systemic diseases, chronic illnesses or clinically significant laboratory abnormalities were excluded from the study. Finally, cats that had received any antibiotics, antacids, anti-inflammatory drugs, or corticosteroids within the past 6 months were excluded.

Cats with clinical signs of chronic enteropathy had presented to the Texas A&M University, Small Animal Hospital, College Station, Texas, or the Veterinary Specialty

Hospital, San Diego, California. Cats with clinical signs of chronic enteropathy (weight loss, hyporexia, vomiting, diarrhea) of ≥ 3 weeks duration were eligible for enrollment. All cats in this group underwent gastro-duodenoscopy and ileo-colonoscopy for diagnostic purposes. Histopathologic examination of H&E stained endoscopic formalin-fixed, paraffin-embedded (FFPE) tissue sections was performed by a single board-certified pathologist (MA) blinded to the clinical status of the cats. In addition, samples underwent immunohistochemistry and PCR for antigen receptor rearrangements (PARR). A final diagnosis of IBD or MEITL was reached upon integration of results from histopathology, immunohistochemistry, and PARR. Cats that had received antibiotics within 4 weeks prior to fecal sampling were excluded from the study.

Spontaneously passed fecal samples were collected from healthy cats, refrigerated and shipped to the Texas A&M Gastrointestinal Laboratory within 24 hours of passing. Fecal samples from cats with CE were either collected after spontaneous void or digitally while the cat was under anesthesia for endoscopy. All samples were shipped on cold packs or on dry ice. Upon arrival, fecal samples were immediately transferred to a lysis buffer and DNA was extracted using the Mobio Power Soil DNA Extraction kit (MoBio Laboratories, Inc., CA) following manufacturer's instructions.

Amplification and sequencing of the V4 variable region of the 16S rRNA gene were performed utilizing the Illumina MiSeq Sequencing platform. Sequencing was performed at MR DNA (Shallowater, TX, USA) following the manufacturer's guidelines using forward and reverse primers: 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'- GGACTACVSGGGTATCTAAT-3'). Briefly, the PCR reaction was

performed in a single-step 28 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles (5 cycles used on PCR products) of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. After sequencing, barcodes and primers were removed from the sequences; then short (<150bp), ambiguous, homopolymeric, and chimeric sequences were depleted from the dataset. Operational Taxonomic Units (OTUs) were assigned based on at least 97% sequence similarity using the QIIME 2.0 pipeline [157]. Sequences assigned as chloroplast, mitochondria and Unassigned were removed before downstream analysis. Additionally, OTUs assigned to the phylum cyanobacteria were considered to be potential plant chloroplast contaminants and excluded from the analysis. Sequences were rarefied to an equal depth of 12,000 sequences per sample. The sequences were deposited in SRA under accession number SRP168128.

**4.3. Statistical analyses**

All datasets were tested for normality using the Shapiro-Wilk test (JMP 10, SAS software Inc.). Differences in bacterial communities between healthy cats and cats with diarrhea were analyzed using the phylogeny-based unweighted UniFrac distance metric, and PCoA plots and rarefaction curves were generated within QIIME 2.0 [157]. ANOSIM (Analysis of Similarity) within the software package PRIMER 6 (PRIMER-E Ltd., Luton, UK) was used to determine significant differences in microbial communities between healthy cats and diseased cats. Because most datasets did not meet the assumptions of normal distribution, statistical testing between healthy and diseased cats

were performed using non-parametric Kruskal-Wallis tests or a Mann-Whitney U test where applicable. The resulting p-values were adjusted for multiple comparisons using the Benjamini & Hochberg's False Discovery Rate (FDR), and an adjusted q < 0.05 was considered statistically significant.[158] A Dunn's post-test was used to determine which disease types differed significantly. Linear discriminant analysis Effect Size (LEfSe) was used to elucidate bacterial taxa (16S rRNA genes) associated with healthy or diseased cats. LEfSe was used online in the Galaxy workflow framework.

The data that support the findings of this study are available from the corresponding author (SM) upon reasonable request.

Ethical approval and informed consent

The study protocol was approved by the Texas A&M University Animal Care and Use Committee (IACUC 2015-0276 CA and IACUC 2014-0369 CA).

## 4.4. Results

### 4.4.1. Animal demographics and clinical activity index

A total of 65 cats were enrolled into this study, 38 healthy cats and 27 cats with chronic enteropathy (13 with IBD and 14 with SCL). A fecal sample was collected from all cats. Demographic characteristics are shown in Table 4.1.

Age did not differ significantly between healthy cats (median age: 9 years, range: 1-15 years) and cats with CE (median age: 10 years, range: 2-16 years; P = 0.052). Cats with CE had a significantly lower body weight (median: 4.6 kg, range: 2.9-10.5 kg) and body condition score (BCS; median: 4, range: 1-9) than healthy cats (median body weight: 5.4 kg, range: 2.5-8.6 kg, median BCS: 5, range: 4-9; P = 0.035 and P < 0.001, respectively). Cats with SCL were significantly older (median age: 11.5 years, range: 7-16 years) than cats with IBD (median age: 8 years, range: 2-16; P = 0.028). Cats with IBD and cats with SCL did not show statistically significant differences with regard to sex, body weight, and BCS. Cats with CE had a median feline chronic enteropathy activity index (FCEAI)[10] score of of 5 (range: 2-11). FCEAI did not differ between cats with IBD (median: 6, range: 3-11) and cats with SCL (median: 5, range: 2-10; p = 0.838).

Table 4.1. Comparison of demographic data between healthy cats and cats with feline chronic enteropathy (FCE).

| | Healthy | Feline CE | p value |
|---|---|---|---|
| number of cats | 38 | 27 | |
| median age in years (range) | 9 (1 – 15) | 10 (2 – 16) | 0.052 |
| median body weight in kg (range) | 5.4 (2.5 – 8.6) | 4.6 (2.9 – 10.5) | 0.035 |
| median body condition score (range) | 5 (4 – 9) | 4 (1 – 9) | <0.0001 |
| sex | 18 FS, 20 MN | 11 FS, 16 MN | 0.596 |
| breeds | 22 DSH, 4 DLH, 2 Maine Coon, 2 Persian, 1 Bombay, 1 Burmese, 1 DMH, 1 Norwegian Forest Cat, 1 Lynx, 1 mixed breed, 1 Sphinx | 17 DSH, 3 DMH, 3 Siamese, 2 DLH, 1 Rag Doll, 1 mixed breed | |

Abbreviations: FS female spayed, MN male neutered, DSH domestic shorthair, DLH domestic longhair, DMH domestic medium hair

## 4.4.2. Sequence analysis and rarefaction

In total, the sequence analysis of the 65 fecal samples yielded 2,837,900 quality sequences (median per sample: 73,741; range: 43,660-145,373).

Alpha diversity at a depth of 43,660 sequences, as described by observed OTUs, Shannon Diversity Index, and Faith Phylogenetic Diversity Index, was significantly lower in cats with CE than in healthy cats ($P = 0.003$, $P = 0.008$, and $P = 0.019$, respectively; Figure 4.1). In addition, alpha diversity appeared to continuously decrease from healthy cats, over cats with IBD to cats with SCL (Results for Kruskal Wallis ANOVA: observed OTUs P = 0.015, Shannon index P = 0.030, Phylogenetic Diversity Index P = 0.049). However, alpha diversity indices did not differ significantly between cats with IBD and cats with SCL. Detailed results for alpha diversity indices are shown in Appendix B. Table 6.3.

Figure 4.1. Alpha diversity indices at a depth of 43,660 sequences.
A. observed OTUs. B. Shannon Diversity Index. C. Faith Phylogenetic Diversity Index. Figures on the left show the comparison between healthy cats and cats with chronic enteropathy (FCE). Figures on the right show subgroups of inflammatory bowel disease (IBD) and small cell lymphoma (SCL).
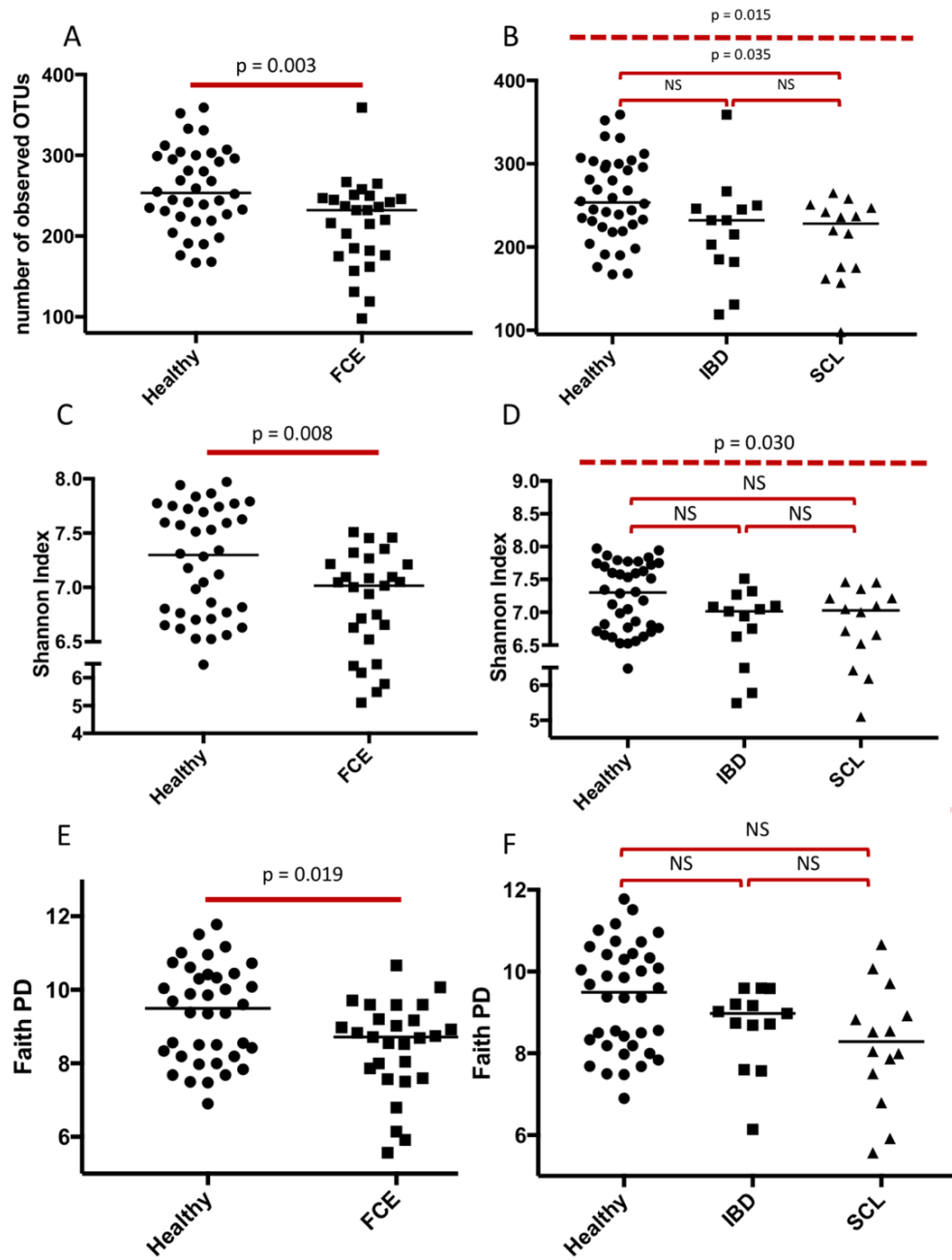
**4.4.3. Microbial communities**

Although cats with CE showed no obvious visible clustering on Principal Component Analysis (PCoA) compared to healthy cats, a statistically significant difference between the two groups was found based on ANOSIM of unweighted Unifrac distances ($P = 0.023$, R = 0.073; Figure 4.2 A). Individual bacterial groups were analyzed using a Kruskal Wallis test. Taxa found to be significantly different before correction for the false discovery rate (FDR) are listed in Table 4.2. Within the phylum *Firmicutes*, bacterial taxa belonging to the family *Ruminococcaceae* (unclassified species of the genus *Oscillospira*) and members of the genus *Turicibacter* (class *Bacilli*, order *Turicibacterales*, family *Turicibacteraceae*) were significantly less abundant in cats with CE than in healthy cats. In addition, cats with CE had significantly decreased bacterial populations belonging to members of the phyla *Bacteroidetes* (one undetermined species and *Bacteroides plebeius*) and *Actinobacteria* (genus *Bifidobacterium*). In contrast, members of the families *Enterobacteriaceae* and *Streptococcaceae* were significantly more abundant in feces from cats with CE. However, although trends were noted, no statistically significant differences were found after correction for FDR. Figure 4.2 B-F depicts results of the statistical analysis for some selected taxa.

Conversely, based on Linear Discriminant Analysis Effect Size (LEfSe), bacteria of the families *Bifidobacteriaceae*, *Ruminococcaceae*, *Turicibacteriaceae*, and *Paraprevotellaceae* were associated with feces from healthy cats, while

*Enterobacteriaceae* and *Streptococcaceae* were associated with those of cats with CE. A

detailed summary of the LEfSe results can be found in Table 4.3.

Figure 4.2. Principal coordinate analysis indicating beta diversity and univariate analysis of selected taxa.
A. Principal coordinate analysis (PCoA) of unweighted UniFrac distances of 16S rRNA genes. Analysis of similarity (ANOSIM) revealed significantly different between healthy cats and cats with chronic enteropathy (*P* = 0.023, R = 0.073), although with a small effect size and no visible clustering. Healthy cats are depicted as blue, cats with inflammatory bowel disease (IBD) as yellow, and cats with small cell lymphoma (SCL) as red dots. (B) – (F): Univariate analysis of relative percent abundance of selected taxa. Members of the family *Ruminococcaceae*, of the genus *Bifidobacterium* and the species *Bacteroides plebeius* were found to be lower in cats with chronic enteropathy (CE) compared to healthy cats. Members of the genus *Streptococcus* and the family *Enterobacteriaceae* were found to be higher in cats with CE compared to healthy cats.
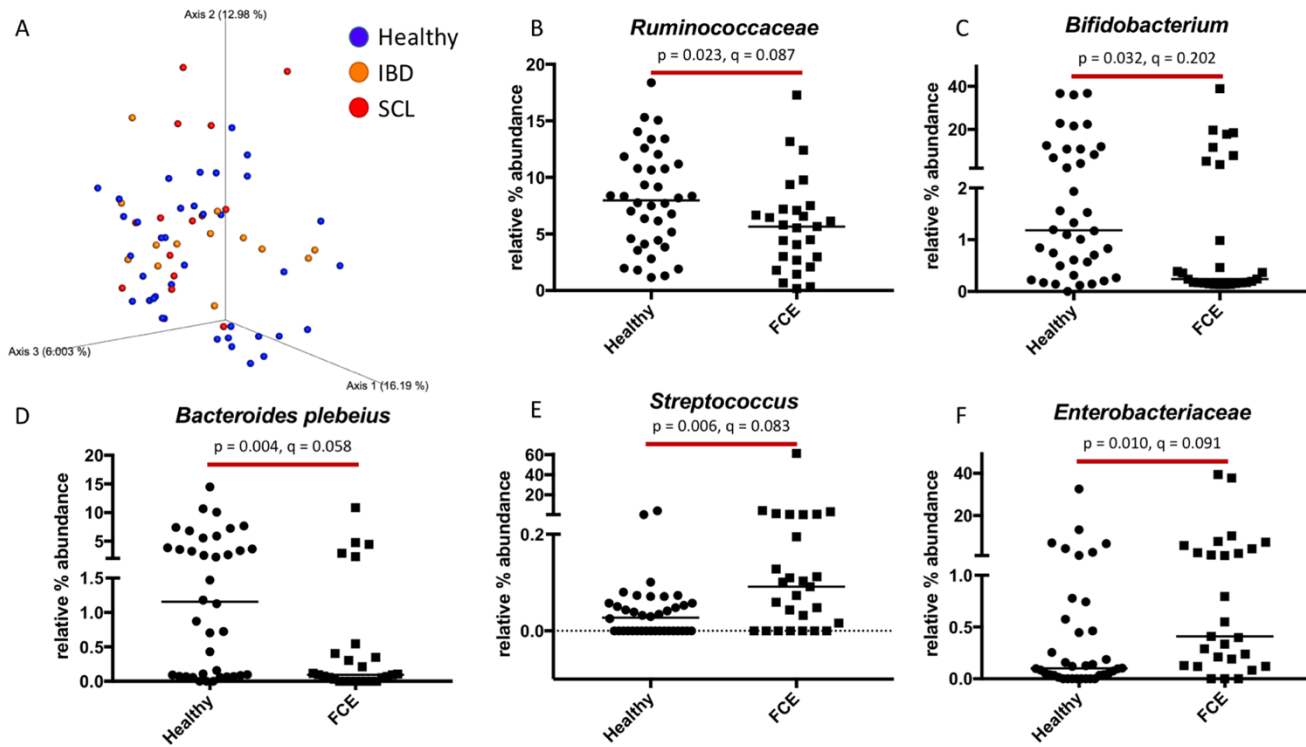
Table 4.2. Taxa found to be significantly different (p-value) between healthy cats and cats with chronic enteropathy (FCE) before correction for false discovery (q value). Numbers represent relative percentages.

| Bacterial Group | Healthy | | FCE | | Healthy vs. FCE | |
|---|---|---|---|---|---|---|
| | | | | | P value | Q value |
| | Median | Range | Median | Range | | |
| **Class** | | | | | | |
| Actinobacteria | 1.2 | 0-36.8 | 0.2 | 0.1-38.9 | 0.0466 | 0.2563 |
| Bacteroidia | 25.3 | 1.2-56.4 | 16.6 | 0.7-55.3 | 0.0458 | 0.2563 |
| **Order** | | | | | | |
| Bifidobacteriales | 1.2 | 0-36.8 | 0.2 | 0.1-38.9 | 0.0316 | 0.14885 |
| Bacteroidales | 25.3 | 1.2-56.4 | 16.6 | 0.7-55.3 | 0.0458 | 0.14885 |
| Turicibacterales | 0 | 0-23.7 | 0 | 0-5.8 | 0.0344 | 0.14885 |
| Enterobacteriales | 0.1 | 0-32.6 | 0.4 | 0-39.4 | 0.0098 | 0.1274 |
| **Family** | | | | | | |
| Bifidobacteriaceae | 1.2 | 0-36.8 | 0.2 | 0.1-38.9 | 0.0316 | 0.16053333 |
| Prevotellaceae | 1.1 | 0-21.7 | 0.5 | 0-22.5 | 0.0519 | 0.1827 |
| Odoribacteraceae | 0 | 0-2.8 | 0.1 | 0-1.4 | 0.0522 | 0.1827 |
| Paraprevotellaceae | 0 | 0-18.5 | 0 | 0-5.6 | 0.0022 | 0.0616 |
| Streptococcaceae | 0 | 0-4.1 | 0.1 | 0-61.4 | 0.0059 | 0.0826 |
| Turicibacteraceae | 0 | 0-23.7 | 0 | 0-5.8 | 0.0344 | 0.16053333 |
| Ruminococcaceae | 8 | 1.2-18.4 | 5.7 | 0.2-17.3 | 0.0228 | 0.1596 |
| Enterobacteriaceae | 0.1 | 0-32.6 | 0.4 | 0-39.4 | 0.0098 | 0.09146667 |
| **Genus** | | | | | | |
| Bifidobacterium | 1.2 | 0-36.8 | 0.2 | 0.1-38.9 | 0.0316 | 0.2021 |
| Prevotella | 0 | 0-18.5 | 0 | 0-5.6 | 0.0182 | 0.17108 |
| Streptococcus | 0 | 0-4.1 | 0.1 | 0-61.4 | 0.0037 | 0.08695 |
| Turicibacter | 0 | 0-23.7 | 0 | 0-5.8 | 0.0344 | 0.2021 |
| Undetermined genus, Peptostreptococcaceae | 0.3 | 0-16.6 | 0.1 | 0-5.9 | 0.04 | 0.20888889 |
| Undetermined genus, Ruminococcaceae | 0.1 | 0-0.9 | 0 | 0-0.5 | 0.0033 | 0.08695 |
| Oscillospira | 1.1 | 0.1-3.5 | 0.7 | 0-2.2 | 0.0181 | 0.17108 |
| Undetermined genus, Erysipelotrichaceae | 0.1 | 0-0.9 | 0 | 0-0.8 | 0.0241 | 0.18878333 |
| Undetermined genus, Enterobacteriaceae | 0.1 | 0-32.6 | 0.4 | 0-39.4 | 0.0081 | 0.1269 |

Table 4.2. Continued. Taxa found to be significantly different (p value) between healthy cats and cats with chronic enteropathy (FCE) before correction for false discovery (q value). Numbers represent relative percentages.

| Bacterial Group | Healthy | | FCE | | Healthy vs. FCE | |
|---|---|---|---|---|---|---|
| | Median | Range | Median | Range | P value | Q value |
| **Species** | | | | | | |
| Undetermined species, Bifidobacterium | 1.1 | 0-35.2 | 0.2 | 0-38.6 | 0.0234 | 0.13737 |
| Undetermined species, Bacteroides | 0.1 | 0-2.3 | 0 | 0-0.8 | 0.0041 | 0.058425 |
| Undetermined species, Bacteroides | 10.8 | 0.3-51 | 6.5 | 0.3-47.4 | 0.3514 | 0.5271 |
| Bacteroides plebeius | 1.2 | 0-14.5 | 0.1 | 0-10.9 | 0.0039 | 0.058425 |
| Undetermined species, Prevotella | 0 | 0-18.5 | 0 | 0-5.6 | 0.0182 | 0.129675 |
| Undetermined species, Enterococcaceae | 0.1 | 0-5 | 0 | 0-1.7 | 0.1968 | 0.37791 |
| Undetermined species, Streptococcus | 0 | 0-4.1 | 0.1 | 0-61.4 | 0.0109 | 0.10355 |
| Undetermined species, Turicibacter | 0 | 0-23.7 | 0 | 0-5.8 | 0.0344 | 0.1691 |
| Undetermined species, Clostridium | 0.2 | 0-10.8 | 0.9 | 0.1-20.3 | 0.0022 | 0.058425 |
| Undetermined species, Ruminococcus | 0 | 0-3.2 | 0.6 | 0-2.5 | 0.0356 | 0.1691 |
| Undetermined species, Peptostreptococcaceae | 0.3 | 0-16.6 | 0.1 | 0-5.9 | 0.04 | 0.17538462 |
| Undetermined species, Ruminococcaceae | 0.1 | 0-0.9 | 0 | 0-0.5 | 0.0033 | 0.058425 |
| Undetermined species, Oscillospira | 1.1 | 0.1-3.5 | 0.7 | 0-2.2 | 0.0181 | 0.129675 |
| Undetermined species, Erysipelotrichaceae | 0.1 | 0-0.9 | 0 | 0-0.8 | 0.0241 | 0.13737 |
| Undetermined species, Enterobacteriaceae | 0.1 | 0-32.6 | 0.4 | 0-39.4 | 0.0081 | 0.09234 |

Table 4.3. Linear discriminant analysis effect size (LEfSE) analysis of 16S sequences. LEfSE was calculated for healthy cats vs. cats with chronic enteropathy (FCE) and in a second step for the subgroups of FCE, inflammatory bowel disease (IBD) and small cell lymphoma (SCL). The Linear Discriminant Analysis Score (LDA) is given as log 10. Only the taxa meeting a significant LDA threshold value of > 2 are shown

| Level | Selected Taxa | Associated Group | LDA Score | Associated Subgroup | LDA Score |
|---|---|---|---|---|---|
| **Phylum** | Bacteroidetes | Healthy | 4.797 | None | NA |
| **Class** | Actinobacteria | Healthy | 4.562 | Healthy | 4.662 |
| | Bacteroida | Healthy | 4.74 | None | NA |
| **Order** | Bifiodobacteriales | Healthy | 4.196 | Healthy | 4.659 |
| | Turicibacterales | Healthy | 4.356 | None | NA |
| | Enterobacteriales | FCE | 4.302 | None | NA |
| | Bacteroidales | Healthy | 4.549 | None | NA |
| **Family** | Streptococcaceae | FCE | 4.229 | SCL | 4.425 |
| | Ruminococcaceae | Healthy | 4.204 | None | NA |
| | Bifidobacteriaceae | Healthy | 4.227 | Healthy | 4.394 |
| | Enterobacteriaceae | FCE | 4.256 | None | NA |
| | Paraprevotellaceae | Healthy | 4.009 | Healthy | 4.116 |
| | Turicibacteraceae | Healthy | 4.04 | None | NA |
| | Odoribacteraceae | None | NA | SCL | 4.289 |
| | Prevotellaceae | None | NA | Healthy | 3.851 |
| **Genus** | Erysipelotrichaceae | Healthy | 3.672 | None | NA |
| | Oscillospira | Healthy | 3.534 | None | NA |
| | Peptostreptococcaceae (unclassified) | Healthy | 3.594 | None | NA |
| | Bifidobacterium | Healthy | 4.293 | Healthy | 4.496 |
| | Turicibacter | Healthy | 3.632 | None | NA |
| | Streptococcus | FCE | 4.119 | SCL | 4.511 |
| | Paraprevotella | Healthy | 3.789 | None | NA |
| | Enterobacteriaceae (unclassified) | FCE | 4.226 | None | NA |
| | Prevotella | Healthy | 4.109 | Healthy | 4.304 |
| | Coriobacteriaceae (unclassified) | None | NA | IBD | 4.187 |

Table 4.3. Continued. Linear discriminant analysis effect size (LEfSE) analysis of 16S sequences. LEfSE was calculated for healthy cats vs. cats with chronic enteropathy (FCE) and in a second step for the subgroups of FCE, inflammatory bowel disease (IBD) and small cell lymphoma (SCL). The Linear Discriminant Analysis Score (LDA) is given as log 10. Only the taxa meeting a significant LDA threshold value of > 2 are shown

| Level | Selected Taxa | Associated Group | LDA Score | Associated Subgroup | LDA Score |
|-------|---------------|------------------|-----------|---------------------|-----------|
| Species | Turicibacter (unclassified) | Healthy | 3.578 | None | NA |
| | Streptococcus (unclassified) | FCE | 4.092 | SCL | 4.494 |
| | Oscillospira (unclassified) | Healthy | 3.509 | None | NA |
| | Bacteroides plebeius | Healthy | 4.069 | Healthy | 4.285 |
| | Enterobacteriaceae (unclassified) | FCE | 4.255 | None | NA |
| | Paraprevotella (unclassified) | Healthy | 3.465 | None | NA |
| | Peptostreptococcaceae (unclassified) | Healthy | 3.652 | None | NA |
| | Bifidobacterium (unclassified) | Healthy | 3.972 | None | NA |
| | Erysipelotrichaceae (unclassified) | Healthy | 3.626 | None | NA |
| | Prevotella copri | None | NA | Healthy | 4.258 |
| | Coriobacteriaceae (unclassified) | None | NA | SCL | 4.169 |

## 4.4.4. Effect of disease subtype on the feline fecal microbiota

Similar to alpha-diversity, beta-diversity showed continuous changes when comparing healthy cats, cats with IBD, and cats with SCL, with sequential increases or decreases of relative percentages between groups. According to Kruskal Wallis tests, the abundance of members of the genus *Bifidobacterium* (class *Actinobacteria*) differed

significantly among the three groups, with highest numbers in healthy cats and lowest numbers in cats with SCL. In contrast, bacteria within the families *Enterobacteriaceae* (phylum *Proteobacteria*) and *Streptococcaceae* (phylum *Firmicutes*) serially increased from healthy cats, to cats with IBD to cats with SCL. However, after correction for the false discovery rate (FDR), none of the differences remained statistically significant. In addition, no significant differences in microbial communities between cats with IBD and cats with SCL were observed. A detailed summary of relative percentages of the most abundant bacterial groups appears in Appendix B, Table 6.4.

## 4.5. Discussion

To our knowledge, this is the first study comparing the fecal microbiome in a cohort of cats with histopathologically confirmed spontaneous CE to that of clinically healthy cats.

Alpha diversity was significantly lower in cats with CE than in healthy cats. In particular, cats with CE tended to show a lower abundance of obligately anaerobic members of the phyla Firmicutes (family *Ruminococcaceae* and *Turicibacteraceae*), Bacteroidetes (e.g. *Bacteroides plebeius* and unclassified species), and Actinobacteria (genus *Bifidobacterium)*. In contrast, facultative anaerobes such as *Enterobacteriaceae* and *Streptococcaceae* tended to be more abundant in cats with CE than in healthy cats. However, although we found differences in abundance of various bacterial taxa between different groups of cats, there were no statistically significant differences after correction for FDR. Nevertheless, the trends found in our cohort of cats mirror common patterns of dysbiosis described in other species such as humans and dogs, i.e. decreased bacterial

79

diversity, decreased members of obligate anaerobes (*Firmicutes* and *Bacteroidetes*) and increased facultative anaerobes (especially of the family of *Enterobacteriaceae*)[71-73,148,150-153]. Dysbiosis has been documented in humans with various enteropathies, such as IBD (ulcerative colitis and Crohn's disease), antibiotic-associated diarrhea, necrotizing enterocolitis, and colorectal cancer[147,154] and also in dogs with chronic enteropathies[159]. Dysbiotic patterns appear to be similar across different forms of enteropathy; however, because the rarity of the disease, there are no studies published on the microbiomes of people with MEITL.

One universal activity of the intestinal microbiome is the metabolism and fermentation of carbohydrates into short chain fatty acids, such as acetate, butyrate, and propionate. Members of the phylum *Firmicutes* are mostly anaerobic and have been shown to exert indirect anti-inflammatory and immune-modulatory effects by producing short chain fatty acids, particularly butyrate[147]. Butyrate is the major energy source of colonocytes[160], thereby contributing to epithelial cell proliferation and repair and to intestinal barrier integrity[161]. In addition, evidence exists that butyrate may exhibit anti-inflammatory and anti-carcinogenic properties[162,163]. The main butyrate-producing bacteria in the human colon are members of the families *Lachnospiraceae* and *Ruminococcaceae* (phylum *Firmicutes*), and their abundance in people is often depleted in dysbiotic states, including UC and CD[150,164]. Our report appears to be the first to show a depletion of butyrate-producing bacteria in cats with CE. Determining the association between the fecal abundance of *Ruminococcaceae* and butyrate concentration in cats with CE might further elucidate the function of this taxon in cats with CE.

Another major hallmark of dysbiosis involves members of the phylum *Bacteroidetes*. *Bacteroidetes* are highly abundant in the healthy microbiome but are decreased in humans and dogs with various forms of enteropathy[72,148,151]. Interestingly, *Bacteroides plebeius* has been found to be strongly associated with remission in people with CD[165]. In our cohort, *Bacteroidetes* and specifically *Bacteroides plebeius* tended to be decreased in cats with CE. Investigating the association between disease outcome and abundance of *Bacteroides plebeius* might be of interest for future studies in cats with CE.

Our study showed a trend towards a decreased abundance of Bifidobacteria in cats with IBD and SCL. Bifidobacteria are also commonly decreased in human patients with IBD[147,166]. Various Bifidobacteria strains have been shown to exert anti-inflammatory properties by regulating immune cells and cytokine networks[167] and by directly and indirectly enhancing intestinal barrier function[168,169]. For instance, *Bifidobacterium* strains have been shown to induce IL-10 producing regulatory T-cells[170,171] and to exert immunoinhibitory effects by interacting with Toll-like receptor-2[172]. Therefore, *Bifidobacteria* have become an attractive therapeutic target and are often used in probiotic formulations[173]. Our study confirms findings of previous studies in cats with enteropathy, where *Bifidobacteriaceae* have previously been found to be decreased[71,75,174], whereas, to the authors' knowledge, such a decrease has not been documented in dogs. This highlights the difference between species and might point toward *Bifidobacteria* as potential therapeutic targets in cats with CE.

Besides a decrease in obligate anaerobic bacteria, dysbiosis is commonly characterized by an increase in facultative anaerobic bacteria, specifically members of the family of *Enterobacteriaceae*[147,175,176]. This phenomenon might be explained by the oxygen gradient model[177,178]. In this model it is hypothesized that during a steady state, the mucosal microbiome is controlled by mucosal immune responses, the intestinal barrier, and competition with luminal bacteria. The intestinal mucosa shows an oxygen gradient, in which the mucosal interface is mostly aerobic while the lumen is mostly anaerobic. It is thought that during inflammation, the intestinal barrier breaks downs, thereby increasing the luminal oxygen tension. This leads to a translocation and expansion of aerotolerant taxa usually located close to the mucosa centripetal into the lumen and centrifugal across the epithelial barrier into the lamina propria, contributing to the inflammatory response[177,178]. Our cohort of cats followed this pattern, with a trend of higher abundance of facultative anaerobic taxa, specifically *Enterobacteriaceae* and *Streptococcaceae*. Both taxa typically consist of facultative anaerobic members and thus might thrive with increased luminal oxygen tension. An increased abundance of *Streptococcaceae* has previously been documented in people and dogs with IBD[179,180].

Our study has several limitations. Although, we found differences in the abundance of various bacterial taxa between different groups of cats, there were no statistically significant differences after correction for FDR, and thus differences did not appear to be as strong as they have been reported for humans and dogs. This phenomenon might be explained by the different disease phenotypes. Whereas diarrhea is the predominant clinical sign of chronic enteropathy in humans[181] and dogs[11,112], it is

less common in cats, in which weight loss, hyporexia, and vomiting are the dominant clinical signs of CE [1,10]. In our cohort of cats, only 5 out of 27 cats showed diarrhea. The reason for the different disease phenotypes in humans, dogs, and cats is not entirely clear, but factors may include different disease localization (i.e. small vs. large bowel) and different disease pathophysiology. Cats often show involvement of the small intestinal tract, but the fecal microbiome represents predominantly bacterial communities present in the distal part of the intestinal tract. In addition, the fecal microbiome might not accurately represent the mucosa-associated microbiome. Therefore, investigating the luminal content within the small intestine or the mucosal microbiome might reveal more distinct differences in microbial communities in cats than this study of fecal samples. However, a recent study comparing the fecal and mucosal microbiomes in human patients with and without IBD found large overlaps between the two microbial habitats in each group[177]. Although it is important to point out that IBD in humans is predominantly a large bowel disease.

We also cannot exclude that a larger sample size would have revealed statistical significant differences after FDR correction.

We did not find any bacterial taxa in which abundance differed significantly between cats with IBD and SCL. However, as a striking pattern many bacterial taxa appeared to either serially increase (e.g., *Enterobacteriaceae*) or decrease (e.g., *Actinobacteria, Bifodobacteria, Prevotella*) when comparing healthy cats, cats with IBD, and cats with SCL. Similarly, Progression of IBD to SCL over months to years has long been suspected, and inflammatory lesions frequently coexist with SCL[31,34]. Our findings

might lend support to the hypothesis that IBD and SCL are not two different diseases but rather a continuum.

In summary, we found that cats with CE show patterns of dysbiosis that have previously been described in people with IBD. Obligately anaerobic taxa in the phyla Firmicutes, Bacteroidetes, and Actinobacteria were depleted in cats with CE, while facultative anaerobes such as *Enterobacteriaceae* and *Streptococcaceae* were more abundant. *Bacteroides plebeius*, a species shown to be associated with positive outcome in people with CD, was decreased in our cohort of cats.

# 5. UNTARGETED METABOLOMIC ANALYSIS IN CATS WITH NATURALLY OCCURRING INFLAMMATORY BOWEL DISEASE AND ALIMENTARY SMALL CELL LYMPHOMA

Feline chronic enteropathy (CE) is a common gastrointestinal disorder in cats and mainly comprises inflammatory bowel disease (IBD) and small cell lymphoma (SCL). We characterized the fecal metabolome of 14 healthy cats and 22 cats with CE (11 cats with IBD and 11 cats with SCL). Principal component- and heat map analysis showed distinct clustering between cats with CE and healthy controls. Random forest classification revealed good group prediction for healthy cats and cats with CE with an overall out of bag error rate of 16.7%. Univariate analysis revealed 84 compounds to be significantly different in cats with CE compared to healthy control cats. Polyunsaturated fatty acids held discriminatory power in differentiating IBD from SCL. Metabolomic profiles of cats with CE showed similarities to CE in people with significant alterations of metabolites that are related to tryptophan, arachidonic acid, and glutathione pathways.

## 5.1. Introduction

Feline chronic enteropathy (CE) is a spontaneously arising disorder in cats that is especially common in the elderly cat population. It is defined as the chronic presence (i.e., longer than 3 weeks) of signs of gastrointestinal disease such as weight loss, vomiting and diarrhea in the absence of infectious intestinal or extraintestinal causes[112]. The most common types of CE in cats are inflammatory bowel disease (IBD) and alimentary small cell lymphoma (SCL)[1,7,12,31]. Diagnosis and differentiation require

invasive and expensive procedures, including the collection and histopathologic examination of intestinal tissue biopsies. Therapeutic strategies generally consist of treatment with immunosuppressive drugs such as corticosteroids for cases of IBD or cytotoxic drugs such as chlorambucil in combination with glucocorticoids for SCL or refractory cases of IBD[1,8,9]. Hence, the discovery of less invasive biomarkers for the diagnosis and differentiation of CE in cats and the identification of new therapeutic targets would be highly desirable.

Previous studies in human subjects revealed global metabolic changes in people with IBD and the potential to use metabolomic profiling for the diagnosis and/or differentiation of IBD and IBD subtypes[87,180,182-185]. Common metabolic perturbations described included pathways affecting tryptophan, amino acids, fatty acids, bile acids, sphingolipids, and biogenic amines[87,180,182-185]. While metabolomic profiles have been reported in plasma[186,187], serum[188] and urine[189] of healthy cats, information from untargeted metabolomic studies in cats with CE are lacking.

Feline alimentary SCL shows some histological parallels to monomorphic epitheliotropic intestinal T-cell lymphoma (MEITL) in people (i.e., formally known as enteropathy associated T-cell lymphoma Type 2 (EATL Type 2)), such as a monomorphic infiltration of the intestinal mucosa with small to medium lymphocytes of predominantly T-cell origin, epitheliotropism, and a predilection for the small intestine[31,38,43]. However, while there are histopathological parallels, the clinical course of feline SCL and EATL Type 2 is very different. While alimentary SCL in cats is

86

slowly progressing with a median survival time of 1.5 to 3.5 years[13,36], MEITL in people is a clinically aggressive disease and generally associated with a poor prognosis[38]. Recently, other authors suggested that the cat might be a suitable model for indolent digestive T-cell lymphoproliferative disorder[17]. This rare disorder in people is characterized by a superficial monoclonal intestinal T-cell infiltrate and the disease is generally indolent or slowly progressive[17,39-43].

We hypothesized that cats with CE have metabolomic perturbations and that metabolomic profiling can distinguish cats with IBD from cats with SCL. We further hypothesized that metabolic perturbances observed in cats with CE would be similar to those observed in dogs and humans with IBD.

## 5.2. Materials and Methods

### 5.2.1. Study approval and enrollment.

This prospective study was conducted at the Veterinary Medical Teaching Hospital, Texas A&M University. The study protocol was approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC 2015-0276 CA and IACUC 2014-0369 CA ). All experiments were performed in accordance with relevant guidelines and regulations. Cat owners provided written informed consent prior study enrollment. Cats with clinical signs of chronic enteropathy (n=22) and control cats (n=14) were recruited from the hospital population at the Small Animal Hospital of Texas A&M University in College Station, Texas or the Veterinary Specialty Hospital in San Diego, California.

Cats with clinical signs of chronic enteropathy (i.e., weight loss, hyporexia, vomiting, diarrhea) of ≥ 3 weeks duration were eligible for enrollment. All cats in this group underwent gastro-duodenoscopy and ileo-colonoscopy for diagnostic purposes. Histopathologic examination of H&E stained endoscopic formalin-fixed, paraffin-embedded (FFPE) tissue sections was performed by a single board-certified pathologist blinded to the clinical status of the cats. In addition, samples underwent immunohistochemistry and PCR for antigen receptor rearrangements (PARR). A final diagnosis of IBD (n=11) or SCL (n=11) was reached upon integration of results from histopathology, immunohistochemistry, and PARR. Cats that had received antibiotics or corticosteroids within 4 weeks prior to fecal sampling were excluded from the study.

Control cats were deemed healthy based on history and physical examination findings. In addition, 12 cats underwent laboratory testing with a complete blood count, serum chemistry profile, and serum concentrations of total T4, cobalamin, folate, feline pancreatic lipase immunoreactivity (fPLI), and feline trypsin-like immunoreactivity (fTLI) to verify the health status. Cats with systemic diseases, chronic illnesses, or clinically significant laboratory abnormalities were excluded from the study. In addition, cats that had received antibiotics, antacids, anti-inflammatory drugs, or corticosteroids within the past 6 months were excluded.

**5.2.2. Sample collection.**

Fecal samples were collected after spontaneous defecation or digitally while the cat was under anesthesia and prepared for endoscopy. Fecal samples were refrigerated or

frozen immediately after collection and shipped to the Gastrointestinal Laboratory at Texas A&M University on ice packs or dry ice within 24 hours. Upon arrival, fecal samples were immediately aliquoted and stored at -80°C until analysis.

### 5.2.3. Metabolite extraction.

Untargeted fecal metabolomic analysis was performed by Metabolon, Inc. (Durham, NC) as previously described [190-192]. Metabolites were extracted from lyophilized and homogenized samples using methanol extraction. Extracts were analyzed by a ACQUITY ultra-performance liquid chromatography (Waters, Milford, CA) and a ThermoFisher Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The scan range covered 70-1000 m/z. Metabolite identification was performed by automated comparison of the ion features in the experimental samples to a reference library. Chemical standard entries included retention time, molecular weight (m/z), preferred adducts, and in-source fragments as well as associated MS spectra, and were curated by visual inspection for quality control using software developed at Metabolon. Peaks were quantified using the area-under-the-curve. Compounds were corrected for inter-day variation by registering the medians to equal one (1.00) and normalizing each data point proportionally. Missing values were imputed with the observed minimum for each compound.

**5.2.4. Statistical Analyses.**

Patient demographics were compared by the Mann-Whitney or Fisher's exact test as appropriate. Differences in the abundance of fecal metabolites between control cats and cats with CE were evaluated using a Mann-Whitney test. A *post hoc* analysis to assess differences in the abundance of metabolites between the subgroups (i.e., controls, IBD, SCL) was performed by Dunn's test. Statistical significance was set at $p < 0.05$. Results were adjusted by False Discovery Rate and reported as the q-value where appropriate. Univariate analysis was performed using Prism 7.0b (Graph Pad Software, La Jolla, CA) and JMP Pro 14.1.0 (SAS Institute Inc., Cary, NC).

Multivariate analysis was performed using MetaboAnalyst[193]. Data was mean centered and divided by the standard deviation of each variable. Principal component analysis (PCA) and hierarchical clustering was performed and a heatmap was created as a visual aid for the dendrogram. Random forest regression analysis was used to evaluate the classification performance of metabolomics.

**Data availability**. The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

**5.3. Results**

Age (p = 0.353) and sex distribution (p > 0.999) were comparable between control cats and cats with CE (Table 5.1). Cats with CE had a significantly lower body weight (p = 0.007) and body condition score[118] (P < 0.001).

When comparing subgroups of cats with CE, cats with SCL were significantly older (median age: 12 years, range: 9-15 years) than cats with IBD (median age: 7 years, range: 2-16; p = 0.028). Cats with IBD and cats with SCL did not show statistically significant differences with regards to sex, body weight, or BCS. Cats with CE had a median feline chronic enteropathy activity index (FCEAI)[10] of 6 (range: 2-11). However, the FCEAI did not differ between cats with IBD (median: 6, range: 3-11) and cats with SCL (median: 5, range: 2-10; p = 0.176).

Table 5.1. Comparison of demographic data between healthy cats and cats with chronic enteropathy (CE).

|  | Healthy | Feline CE | P value |
|---|---|---|---|
| **Number of cats** | 14 | 22 | |
| **Median age in years (range)** | 8.5 (3-15) | 10.5 (2-16) | 0.353 |
| **Median BW in kg (range)** | 5.5 (3.9-8.0) | 4.6 (2.5-7.64) | 0.007 |
| **Median BCS (range)** | 5.5 (5-9) | 4 (1-9) | <0.001 |
| **Sex** | 18 FS, 20 MN | 11 FS, 16 MN | >0.999 |
| **Breeds** | 13 DSH, 1 mixed breed | 6 DSH, 2 DMH, 2 DLH, 1 mixed breed | |

Abbreviations: BCS[118]= body condition score, 1-4: underweight, 5: ideal, 6-9 overweight; BW = body weight; DLH = domestic longhair; DMH = domestic medium hair; DSH = domestic shorthair, FS = female spayed, MN = male neutered

A total of 856 named metabolites were detected. Nonparametric univariate analysis using a FDR of 5% revealed a total of 84 metabolites that differed significantly between control cats and cats with CE (Table 5.2). PCA, hierarchical clustering and a heatmap revealed compounds that differed significantly between control cats and cats with CE indicated clustering of the two cohorts (Figure 5.1 a, b) . Random forest classification showed a good group prediction, with a 16.7% out of bag (OBB) error rate (Table 5.3).

Table 5.2. Metabolites and pathways significantly altered in cats with chronic enteropathy (CE).
[a]↓ indicated downregulation and ↑ indicates upregulation compared with findings in healthy control cats. Fold change was calculated for cats with CE relative to healthy cats

| Class/pathway and metabolites | | P-value | Q-value | Fold change direction (↑ or ↓) and magnitude |
|---|---|---|---|---|
| Sub-pathway | Metabolite | | | |
| Amino acids and metabolites | | | | |
| Alanine and aspartate metabolism | Alanine | 0.0043 | 0.0470 | ↑2.2936 |
| | Aspartate | 0.0022 | 0.0346 | ↑2.7186 |
| Glutathione metabolism | Gamma-glutamylglutamine | 0.0014 | 0.0331 | ↑5.3251 |
| | 2-hydroxybutyrate/2-hydroxyisobutyrate | 0.0004 | 0.0228 | ↑9.0087 |
| Glycine, serine and threonine metabolism | 2-methylserine | 0.0015 | 0.0331 | ↑3.5909 |
| | Glycine | 0.0035 | 0.0445 | ↑2.2987 |
| Guanidino and acetamido metabolism | Guanidinosuccinate | 0.0014 | 0.0331 | ↑3.6303 |
| Leucine, Isoleucine and Valine Metabolism | 3-methylglutaconate | 0.0022 | 0.0346 | ↓0.3917 |
| | Alpha-hydroxyisovalerate | 0.0022 | 0.0346 | ↑4.804 |
| | Isoleucine | 0.0043 | 0.0470 | ↑2.2281 |
| | Leucine | 0.0028 | 0.0383 | ↑2.3409 |
| | Valine | 0.0021 | 0.0347 | ↑2.9777 |
| Methionine, cysteine, SAM and taurine metabolism | Cysteine sulfinic acid | 0.0007 | 0.0258 | ↑2.9493 |
| Phenylalanine metabolism | Phenylalanine | 0.0039 | 0.0470 | ↑2.4088 |
| Tryptophan metabolism | 5-hydroxyindoleacetate | 0.0011 | 0.0327 | ↓0.4893 |
| | 2-oxindole-3-acetate | <0.0001 | 0.0089 | ↓0.1789 |
| | Indole-3-carboxylic acid | 0.0023 | 0.0347 | ↑2.1828 |

Table 5.2. Continued. Metabolites and pathways significantly altered in cats with chronic enteropathy (CE).
a↓ indicated downregulation and ↑ indicates upregulation compared with findings in healthy control cats. Fold change was calculated for cats with CE relative to healthy cats

| Class/pathway and metabolites | | P-value | Q-value | Fold change direction (↑ or ↓) and magnitude |
|---|---|---|---|---|
| Sub-pathway | Metabolite | | | |
| Amino acids and metabolites | | | | |
| Tyrosine metabolism | 3-(4-hydroxyphenyl)lactate | 0.0025 | 0.0361 | ↑19.648 |
| | Gentisate | 0.0016 | 0.0331 | ↓0.3671 |
| Carbohydrate | | | | |
| Pentose metabolism | Xylose | 0.0022 | 0.0347 | ↓0.2278 |
| Cofactors and vitamins | | | | |
| Nicotinate and nicotinamide metabolism | N1-Methyl-4-pyridone-3-carboxamide | 0.0001 | 0.0155 | ↑4.6074 |
| Tocopherol metabolism | Alpha-CEHC | 0.0015 | 0.0331 | ↑ 7.7239 |
| | Delta-tocopherol | 0.0039 | 0.0470 | ↑2.3009 |
| Vitamin A metabolism | Beta-cryptoxanthin | 0.0003 | 0.0228 | ↓0.2144 |
| | Carotene diol (1) | 0.0005 | 0.0228 | ↓0.3609 |
| | Carotene diol (2) | 0.0005 | 0.0228 | ↓0.2826 |
| | Carotene diol (3) | 0.0003 | 0.0228 | ↓0.2709 |

Table 5.2. Continued. Metabolites and pathways significantly altered in cats with chronic enteropathy (CE).
a↓ indicated downregulation and ↑ indicates upregulation compared with findings in healthy control cats. Fold change was calculated for cats with CE relative to healthy cats

| Class/pathway and metabolites | | P-value | Q-value | Fold change direction (↑ or ↓) and magnitude |
|---|---|---|---|---|
| Sub-pathway | Metabolite | | | |
| Lipids | | | | |
| Ceramide | Ceramide (d16:1/24:1, d18:1/22:1) | 0.0045 | 0.0490 | ↑8.3153 |
| | Ceramide (d18:1/14:0, d16:1/16:0) | 0.0013 | 0.0331 | ↑5.2335 |
| | N-palmitoyl-sphingadienine (d18:2/16:0) | 0.0023 | 0.0347 | ↑5.5645 |
| | N-palmitoyl-sphingosine (d18:1/16:0) | 0.0043 | 0.0470 | ↑3.3205 |
| Fatty acid, hydroxyl | LAHSA (18:2/OH-18:0) | 0.0003 | 0.0228 | ↑35.365 |
| | OAHSA (18:1/OH-18:0) | 0.0002 | 0.0228 | ↑37.144 |
| | PAHSA (16:0/OH-18:0) | 0.0005 | 0.0228 | ↑32.316 |
| Fatty acid, branched | 12-methyltridecanoate (i14:0) | 0.0005 | 0.0228 | ↓0.3418 |
| Fatty acid, dicarboxylate | Adipate (C6-DC) | 0.0017 | 0.0341 | ↑2.7411 |
| | Azelate (C9-DC) | 0.0014 | 0.0331 | ↓0.3523 |
| | Dodecenedioate (C12:1-DC) | 0.0031 | 0.0413 | ↑2.2159 |
| | Octadecenedioate (C18:1-DC) | 0.0005 | 0.0228 | ↑3.3075 |
| | Pimelate (C7-DC) | 0.0043 | 0.0470 | ↓0.46686 |
| | Sebacate (C10-DC) | 0.001 | 0.0327 | ↓0.3776 |

Table 5.2. Continued. Metabolites and pathways significantly altered in cats with chronic enteropathy (CE). a↓ indicated downregulation and ↑ indicates upregulation compared with findings in healthy control cats. Fold change was calculated for cats with CE relative to healthy cats

| Class/pathway and metabolites | | P-value | Q-value | Fold change direction (↑ or ↓) and magnitude |
|---|---|---|---|---|
| Sub-pathway | Metabolite | | | |
| Fatty acid, monohydroxy | 3-hydroxyoleate | 0.0016 | 0.0331 | ↑4.8978 |
| | 3-hydroxysuberate | 0.0053 | 0.0511 | ↑1.7385 |
| | 10-hydroxystearate | <0.0001 | 0.0149 | ↑5.766 |
| | 13-HODE + 9-HODE | 0.0011 | 0.0327 | ↑2.0752 |
| Fatty acid metabolism(acyl carnitine) | Oleoylcarnitine (C18:1) | 0.0025 | 0.0361 | ↑4.2787 |
| | Palmitoylcarnitine (C16) | 0.0042 | 0.0470 | ↑4.6424 |
| Long chain fatty acid | 10-heptadecenoate (17:1n7) | 0.0043 | 0.0470 | ↑5.9203 |
| | 10-nonadecenoate (19:1n9) | 0.0025 | 0.0361 | ↑11.735 |
| | Eicosenoate (20:1) | 0.0008 | 0.0287 | ↑12.345 |
| | Erucate (22:1n9) | 0.0013 | 0.0331 | ↑12.773 |
| | Myristate (14:0) | 0.0011 | 0.0327 | ↑5.9909 |
| | Oleate/vaccenate (18:1) | 0.0031 | 0.0413 | ↑5.6274 |
| Lysophospholipid | 1-stearoyl-GPC (18:0) | 0.0035 | 0.0445 | ↑13.363 |
| Lysoplasmalogen | 1-(1-enyl-oleoyl)-GPE (P-18:1) | 0.0007 | 0.0258 | ↑7.8633 |
| Medium chain fatty acids | Laurate (12:0) | 0.0005 | 0.0228 | ↑11.522 |

Table 5.2. Continued. Metabolites and pathways significantly altered in cats with chronic enteropathy (CE).
a↓ indicated downregulation and ↑ indicates upregulation compared with findings in healthy control cats. Fold change was calculated for cats with CE relative to healthy cats

| Class/pathway and metabolites | | P-value | Q-value | Fold change direction (↑ or ↓) and magnitude |
|---|---|---|---|---|
| Sub-pathway | Metabolite | | | |
| Polyunsaturated fatty acids (n3 and n6) | Arachidonate (20:4n6) | 0.0025 | 0.0361 | ↑7.9203 |
| | Docosadienoate (22:2n6) | 0.0018 | 0.0345 | ↑8.8555 |
| | Docosahexaenoate (DHA; 22:6n3) | 0.0048 | 0.0499 | ↑18.558 |
| | Docosatrienoate (22:3n3) | 0.0028 | 0.0383 | ↑7.0842 |
| | Heneicosapentaenoate (21:5n3) | 0.0023 | 0.0347 | ↑12.74 |
| | Hexadecatrienoate (16:3n3) | 0.0028 | 0.0383 | ↑6.5171 |
| | Nisinate (24:6n3) | 0.0011 | 0.0327 | ↑10.845 |
| Sphingomyelins | Behenoyl sphingomyelin (d18:1/22:0) | 0.0040 | 0.0470 | ↑24.455 |
| | Sphingomyelin (d17:1/16:0, d18:1/15:0, d16:1/17:0) | 0.0001 | 0.0155 | ↑9.9925 |
| | Sphingomyelin (d18:1/14:0, d16:1/16:0) | 0.0004 | 0.0228 | ↑10.726 |
| | Sphingomyelin (d18:2/16:0, d18:1/16:1) | 0.0040 | 0.0470 | ↑4.4038 |
| Sphingosines | Hexadecasphingosine (d16:1) | 0.0048 | 0.0499 | ↑3.4953 |
| Sterols | Beta-sitosterol | 0.0005 | 0.0228 | ↓0.3931 |
| | Campesterol | 0.0039 | 0.0470 | ↑3.1733 |
| | Ergosterol | 0.0016 | 0.0331 | ↓0.357 |
| | Fucosterol | 0.0020 | 0.0347 | ↓0.3795 |
| | Stigmasterol | <0.0001 | 0.0089 | ↓0.3387 |

97

Table 5.2. Continued. Metabolites and pathways significantly altered in cats with chronic enteropathy (CE).
a↓ indicated downregulation and ↑ indicates upregulation compared with findings in healthy control cats. Fold change was calculated for cats with CE relative to healthy cats

| Class/pathway and metabolites | | P-value | Q-value | Fold change direction (↑ or ↓) and magnitude |
|---|---|---|---|---|
| Sub-pathway | Metabolite | | | |
| Nucleotide | | | | |
| Pyrimidine metabolism, cytidine containing | 5-hydroxymethylcytosine | 0.0019 | 0.0346 | ↑7.6263 |
| Pyrimidine metabolism, thymine containing | Thymine | 0.0016 | 0.0331 | ↑2.2599 |
| Xenobiotics | | | | |
| Benzoate metabolism | 3-(3-hydroxyphenyl)propionate | <0.0001 | 0.0089 | ↓0.1316 |
| Chemical | 4-Acetamidobenzoate | 0.0004 | 0.0228 | ↓0.2582 |
| Drug - topical agents | Salicylate | 0.001 | 0.0327 | ↓0.3601 |
| Food component/plant | 3-hydroxycinnamate | 0.0005 | 0.0228 | ↑2.4750 |
| | DIMBOA | <0.0001 | 0.0089 | ↓0.2167 |
| | Equol | 0.0016 | 0.0331 | ↑51.443 |
| | Gluconate | 0.0022 | 0.0347 | ↑7.674 |
| | Vanillin | 0.0048 | 0.0499 | ↑2.3458 |
| Xanthine Metabolism | 1-methylurate | 0.0017 | 0.0342 | ↓0.3717 |

Figure 5.1. Multivariate analysis of the fecal metabolome of healthy cats and cats with chronic enteropathy.
(A) Heat map showing metabolites that were significantly different between healthy cats and cats with inflammatory bowel disease (IBD) and alimentary small cell lymphoma (SCL). Groups are represented by the colored bars at the top of the figure as red (healthy, n=14), blue (IBD, n=11), and green (SCL, n=11). Clusters can be identified between healthy cats and cats with feline chronic enteropathy (FCE) but not between the disease subgroups IBD and SCL. (B) PCA score plots of metabolites in feces from healthy cats (green) and cats with feline chronic enteropathy (FCE, red). (C) PCA score plots of metabolites in feces from healthy cats (red), cats with inflammatory bowel disease (blue), and cats with alimentary small cell lymphoma (green). A cluster can be identified for healthy cast but no specific clusters can be seen for the subgroups of IBD and SCL. Data was mean centered and divided by the standard deviation of each variable (autoscaled)
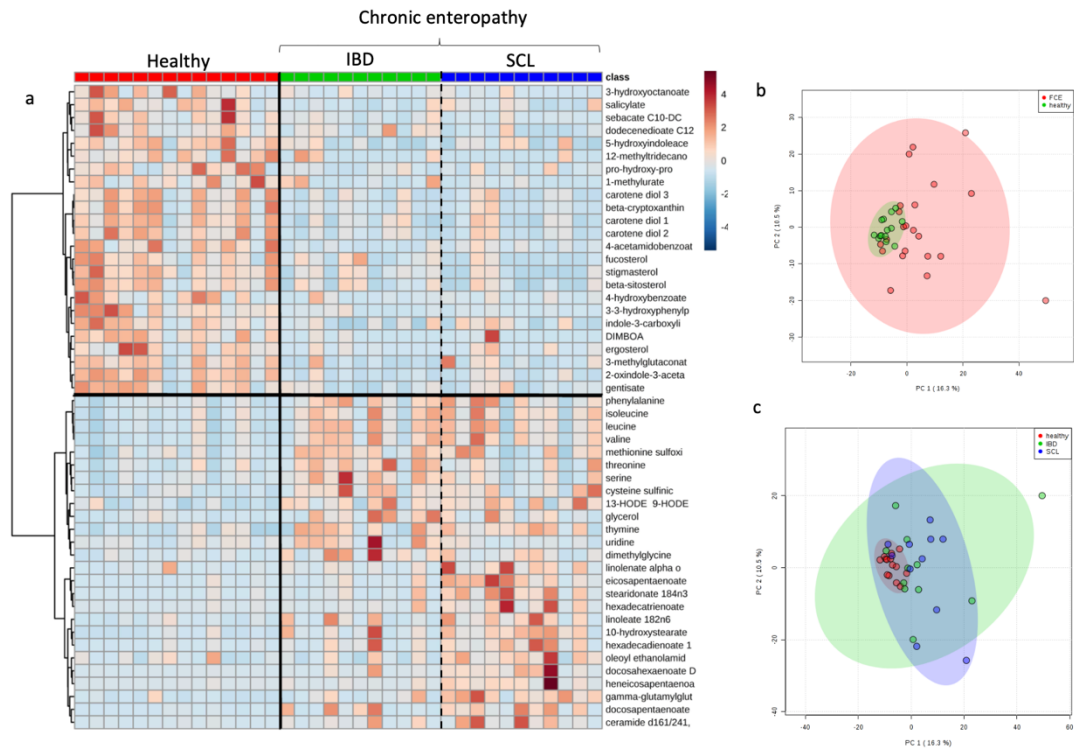
Table 5.3. Random Forest Classification into healthy cats or cats with feline chronic enteropathy (FCE). Overall out of bag (OBB) error rate is 16.7%

| | Predicted group | | |
|---|---|---|---|
| Actual group | FCE | Healthy | Class error* |
| FCE | 21 | 1 | 0.05 |
| Healthy | 5 | 9 | 0.36 |

The random forest importance plot identified 7 metabolites key in classifying the data with sphingomyelin (d18:1/14:0, d16:1/16:0), 3-(3-hydroxyphenyl)propionate, beta-cryptoxanthin, myristoleate (14:1n5), N1-Methyl-4-pyridone-3-carboxamide, 2-oxindole-3-acetate, 5-hydroxyindoleacetate, having the most influence on classification (Figure 5.2).

A complete list of identified metabolites and their comparison between healthy cats and cats with CE can be found in Appendix C, Table 6.5 and Table 6.6Table 6.6.

Figure 5.2 Random Forest variable importance plot and scatter plots of the top seven metabolites with the highest importance for model accuracy. (a) Random Forest variable importance plot. Random Forest is a supervised, machine learning algorithm used for regression (prediction) and classification analysis of large data sets. This Random Forest algorithm was based on the comparison of cats with feline chronic enteropathy (FCE) and healthy control cats. The model identifies features with the highest predictive accuracy for health status. The variable importance plots shows the significant features identified by Random Forest. The features are ranked by the mean decrease in classification accuracy when they are permuted. A higher value indicates the importance of that metabolite in predicting the group (healthy vs. chronic enteropathy). Data was mean centered and divided by the standard deviation of each variable (autoscaled). (b) Scatterplots of the top seven metabolites from (a).
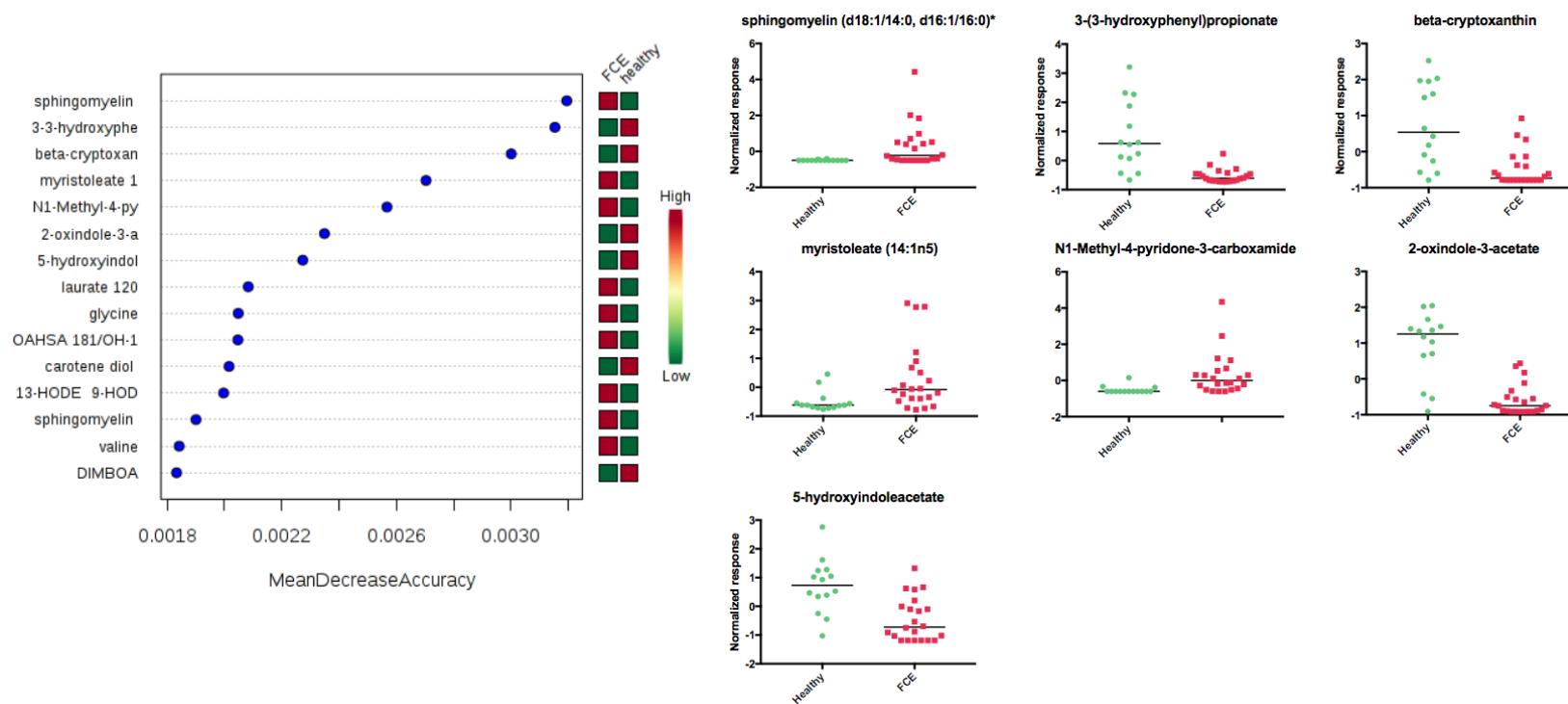
Amongst biochemicals found to be increased in feces from cats with CE were various amino acids and their metabolites (e.g., aspartate, cysteine sulfinic acid, phenylalanine, leucine, and valine), fatty acids (e.g., arachidonate and eicosanoids), metabolites within vitamin A metabolism (e.g., carotendiols), and simple sphingolipids (e.g., ceramide, sphingomyelins, and sphingosines). Compounds that revealed a significantly lower abundance in cats with CE compared to healthy controls were part of the tryptophan metabolism (i.e., indole-derivates), vitamin A metabolism (i.e., carotendiols), and sterols.

Upon analysis of the CE subgroups, IBD and SCL, multivariate analysis revealed no visible clustering between cats with IBD and cats with SCL (Figure 5.1, a, c). Random forest analysis revealed poor group prediction with an OBB of 47.2% and class error rates of 82% and 55% for cats with IBD and cats with SCL, respectively. However, univariate analysis revealed 18 metabolites to be significantly different between healthy cats, cats with IBD, and cats with SCL. *Post hoc* analysis revealed 3 polyunsaturated fatty acids (i.e., eicosapentaenoate, heneicosapentaenoate, and stearidonate) within the eicosanoid family to be different between cats with IBD and cats with SCL (Table 5.4).

Table 5.4. Significance of metabolites for the discrimination between the three groups, healthy controls, cats with inflammatory bowel disease (IBD), and cats with alimentary small cell lymphoma (SCL).
[a]Kruskal-Wallis test, three groups. [b]Dunn's post hoc. [c] $0.01 < P < 0.05$. [d]$0.001 < P < 0.01$. [e] $P < 0.001$. Not significant = ns

| Subpathway | Metabolite | P value[a] | Q value | Multiple comparison test[b] | | |
| --- | --- | --- | --- | --- | --- | --- |
| | | | | Healthy vs IBD | Healthy vs. SCL | IBD vs. SCL |
| *Amino acids* | | | | | | |
| Glutamine | Gamma-glutamylglutamine | 0.0004 | 0.0307 | ns | SCL > H[e] | ns |
| Tryptophan | 2-oxindole-3-acetate | 0.0002 | 0.0282 | H > IBD[e] | H > SCL[d] | ns |
| *Cofactors and Vitamins* | | | | | | |
| Nicotinate and nicotinamide metabolism | N1-Methyl-4-pyridone-3-carboxamide | 0.0003 | 0.0307 | IBD > H[c] | SCL > H[e] | ns |
| Lipid | | | | | | |
| Fatty acid, hydroxyl | LAHSA (18:2/OH-18:0) | 0.0004 | 0.0307 | ns | SCL > H[e] | ns |
| | OAHSA (18:1/OH-18:0) | 0.0004 | 0.0307 | ns | SCL > H[e] | ns |
| | PAHSA (16:0/OH-18:0) | 0.0006 | 0.0345 | ns | SCL > H[e] | ns |
| Fatty acid, monohydroxy | 10-hydroxystearate | 0.0002 | 0.0282 | IBD > H[c] | SCL > H[e] | ns |
| Polyunsaturated fatty acid | Heneicosapentaenoate (21:5n3) | 0.0002 | 0.0282 | ns | SCL > H[e] | SCL > IBD[c] |
| | Stearidonate (18:4n3) | 0.0004 | 0.0307 | ns | SCL > H[e] | SCL > IBD[d] |
| | Eicosapentaenoate (EPA; 20:5n3) | 0.0006 | 0.0345 | ns | SCL > H[e] | SCL > IBD[c] |
| | Hexadecatrienoate (16:3n3) | 0.0006 | 0.0345 | ns | SCL > H[e] | ns |
| | Nisinate (24:6n3) | 0.0008 | 0.0394 | ns | SCL > H[e] | ns |
| Sphingomyelins | Sphingomyelin (d17:1/16:0, d18:1/15:0, d16:1/17:0) | 0.0002 | 0.0282 | IBD > H[c] | SCL > H[e] | ns |
| | Sphingomyelin (d18:1/14:0, d16:1/16:0) | 0.0005 | 0.0307 | ns | SCL > H[e] | ns |
| Sterol | Stigmasterol | 0.0001 | 0.0282 | H > IBD[c] | H > SCL[e] | ns |
| *Xenobiotics* | | | | | | |
| Benzoate metabolism | 3-(3-hydroxyphenyl)propionate | 0.0002 | 0.0282 | H > IBD[d] | H > SCL[e] | ns |
| Chemical | 4-acetamidobenzoate | 0.0009 | 0.0435 | H > IBD[e] | ns | ns |
| Food component/plant | DIMBOA | 0.0002 | 0.0282 | H > IBD[e] | H > SCL[d] | ns |

**5.4. Discussion**

Our study revealed global metabolic changes in cats with chronic enteropathy compared to healthy controls with many metabolic pathways affected. Both, hierarchical cluster analysis and PCA, revealed clustering among cats diagnosed with chronic enteropathy and healthy control cats. Random forest analysis showed a good class prediction of 80.6%. Univariate analysis showed a total of 84 metabolites to be significantly different between both groups after controlling for a 5% FDR. Similar studies in humans with inflammatory bowel disease have also shown significant metabolomic differences between affected patients and healthy controls. While IBD in humans and CE in cats share only few characteristics, the utilization of metabolomics data in humans with chronic enteropathies would support this type of approach in further assessing cats with various forms of CE. In addition, metabolic consequences of feline CE and human IBD appear to be very similar. Metabolites or metabolite families commonly found to be affected by IBD in humans are amino acids[87,88,180,183-185], bile acids[183], fatty acids[183,184], and metabolites of the tryptophan pathway[183,194].

Our study revealed multiple amino acids to be increased in the feces from cats with CE, indicating malabsorption, likely as consequence of mucosal inflammation and/or neoplastic infiltration. Interestingly, 2-hydroxybutyrate/2-hydroxyisobutyrate and gamma-glutamylglutamine, two metabolites related to the glutathione-metabolism, were found to be significantly higher in feces from cats with CE based on both uni- and multivariate analysis. Gamma-glutamyl amino acids are precursors for the formation of glutathione in the gamma-glutamyl cycle (glutathione salvage pathway)[195] (Figure 5.3).

Reactive oxygen species have been implicated to contribute to tissue injury in patients with Crohn's disease and ulcerative colits[196,197]. Glutathione is the major intracellular antioxidant, and thus a critical part of the defense mechanism against oxidative stress in inflammatory conditions such as CE[198,199]. Consequently, glutathione precursors are in high demand during catabolic conditions and corresponding higher loads of oxidative stress[88]. Increased fecal concentrations of gamma-glutamyl glutamine in cats with CE might indicate increased loss and correspond to a decreased mucosal availability and glutathione synthesis.

Figure 5.3. Glutathione salvage pathway.
This figure details the metabolism of gamma-glutamyl amino acids to glutathione, a major cellular defense mechanism of oxidative stress.
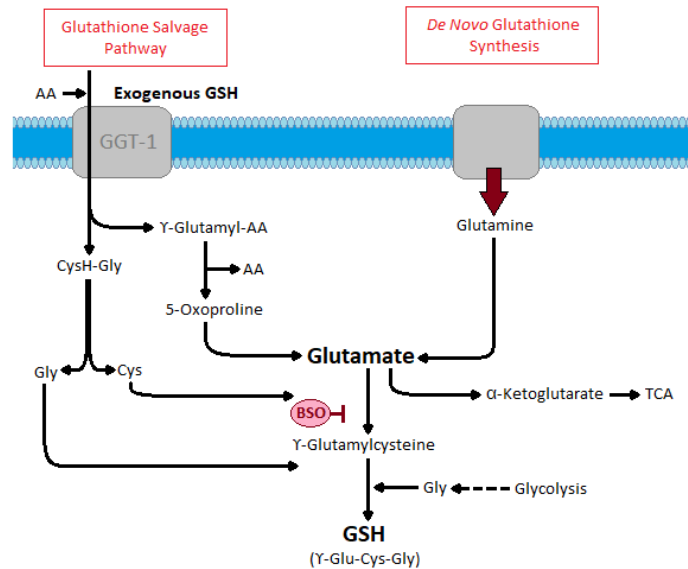
Our study revealed arachidonate to be increased in feces from cats with CE. Arachidonate is a well described mediator of inflammation, including intestinal inflammation, and precursor for prostaglandins essential immune signaling molecules[200]. In addition, arachidonate has been found to increase the expression of intercellular adhesion molecule 1 (ICAM-1)[201]. Adhesion molecules such as ICAM-1 and mucosal addressin cell adhesion molecule (MAdCAM) are involved in the recruitment of leucocytes to the site of inflammation and have been identified as therapeutic targets for human patients with IBD[202]. Increased fecal arachidonate may reflect mucosal upregulation during inflammation and subsequent leakage into the fecal stream. On the other hand, omega-3 polyunsaturated fatty acids, including eicosapentaenoate, have been found to be significantly higher in the SCL subgroup of cats with CE compared to healthy controls.

Eicosapentanoate and other omega-3 fatty acids have anti-inflammatory properties and inhibit conversion of arachidonate into the pro-inflammatory thromboxane-2 and prostaglandin-2 families[203]. Increased fecal levels of omega-3 fatty acids might indicate an increased loss or malabsorption and thus may correspond to the increased arachidonate concentration found in this study.

This study also found several metabolites of the indole family, and pathway to be altered in cats with CE. Amongst a wide range of structurally divergent exo- and endogenic chemicals[204], indole derivates are important ligands for the acyl hydrocarbon receptor (AhR)[205]. AhR signaling is considered an integral component of intestinal

mucosal homeostasis by acting on innate and adaptive immune cells as well as on epithelial renewal and mucosal barrier function[206]. Tryptophan plays a central role in AhR activation because it is transformed into indole and indole derivates by the gut microbiota. Studies in murine and porcine models of colitis found significantly reduced disease activities after oral supplementation with indole-3-propionate or L-tryptophan through the activation of anti-inflammatory pathways mediated by IL-10 and IL-22[205,207]. We found indole derivates, such as 2-oxindole-3-acetate and 5-hydroxyindoleacetate, to be significantly decreased in feces from cats with CE. Although, tryptophan did not significantly differ between CE and control cat, it showed a trend (p=0.0855, q=0.2385) towards a higher fecal excretion in cats with CE[205,208,209] (Appendix C, Table 6.5 and Table 6.6). These findings might indicate a disrupted transformation of tryptophan into indole derivates, possibly linked to intestinal dysbiosis in this cohort of cats with CE.

Finally, our study revealed increased concentrations of several simple sphingolipids in feces from cats with CE. The role of sphingolipids in IBD is complex and incompletely understood. It appears however, that a functional equilibrium between simple sphingolipids (e.g., sphingomyelin, sphingosine, ceramide) and complex sphingolipids (e.g., gangliosides GM3 and GD3) is essential in maintaining intestinal homeostasis[210]. Proinflammatory sphingolipid patterns have previously been described, with increased concentrations of sphingomyelin and ceramide in feces of animal models of colitis[211,212] as well as in the ileal mucus of patients with CD[33] indicating that

sphingomyelin and ceramide accompany and possibly aggravate chronic intestinal inflammation.

Fecal extracts obtained from cats with IBD and SCL revealed similar global changes in metabolic profiles. However, changes observed in cats with IBD were less pronounced as those observed in cats with SCL. This suggests that metabolic consequences are more severe in SCL compared to IBD. In particular, higher concentrations of polyunsaturated fatty acids of the eicosanoid family were found in SCL.

This study has several limitations. Inter-individual variation of metabolomic profiles have been described in humans, dogs, and cats and might be related to a variety of exogenous and endogenous factors such as the environment, diet, gut microbiota, xenobiotics, and the genome[186,189,213]. Our study aimed to characterize clinically relevant changes in the fecal metabolome of cats with CE when compared to healthy subjects. We controlled for demographic characteristics such as age, sex, and breed, where no differences were found between groups. Environmental factors, such as housing and diet were not controlled in this study as it would have made the results clinically irrelevant. This concept is supported by recent studies showed that standardization in is a major source of poor reproducibility preclinical trials[214,215]. Although, we cannot exclude that these factors confounded our results, most are in line with findings across different species with spontaneous or induced IBD and thus likely reflect true changes of the fecal metabolome. In addition, most cats in both groups were housed indoors and fed a variety

of different commercial diets. Another limitation is the limited number of animals in this study. However, untargeted "omics" analysis usually provide only relative changes between subjects or groups and are generally conducted using a smaller sample size. Therefore, all untargeted analyses have to be followed by targeted assays to confirm results of those fishing expeditions on a larger number of subjects. However, this was beyond the scope of this study.

In summary, our study revealed global metabolic changes in cats with CE compared to healthy controls. Many metabolic pathways were affected such as amino acids, fatty acids (e.g., arachidonic acid and eicosanoids), sphingolipids, and metabolites of the tryptophan pathway (i.e., indole-derivates). Metabolic profiles are similar to patterns found in humans and other animal models with IBD and thus metabolic consequences of feline CE and human. Future studies investigating the mucosal and serum metabolome of cats with CE should be conducted to further elucidate the origin of metabolic perturbances and allow further insights into disease pathogenesis. In addition, targeted analysis of the compounds found to be altered in cats with CE is indicated to confirm results and to investigate their value as non-invasive biomarkers

# 6. CONCLUSIONS

## 6.1. Histopathology, immunohistochemistry, and molecular clonality testing of small intestinal biopsy specimens in clinically healthy client-owned cats

Histopathology, immunohistochemistry, and molecular clonality testing are currently considered the gold standard for the diagnosis and differentiation of feline IBD from SCL[12,31]. However, histopathological standards for the diagnosis of gastrointestinal inflammation in endoscopic biopsy specimens from cats published by the WSAVA were developed using full-thickness biopsy specimens collected from young and/or SPF cats[24,25]. This is in contrast to the population of cats that is affected by CE, which are commonly middle-aged to older[4,10,14]. Clinically healthy client-owned cats with demographic characteristics similar to cats with CE frequently showed histopathologic changes that are considered to be abnormal based on current WSAVA standards. A high-interobserver variability has previously been reported[26] and remained even after standardization criteria were formulated by the WSAVA working group in 2008[27]. A possible approach to eliminate observer-bias could be to establish standardized computer-assisted methods to assess histopathologic changes of the intestinal mucosa in cats with feline CE[216].

PCR-based clonality assays, such as PARR, are widely available and can be performed on formalin-fixed paraffin-embedded tissue[34]. Therefore, PARR became the most commonly used method for the differentiation of feline IBD from SCL[12]. Some previous publications have suggested that reclassification of cases based on results of

PARR should be considered[7,217]. However, the sensitivity and specificity of these assays have been reported to be a significant problem in human medicine and led to the formation of a EuroClonality consortium and the development of standardized multiplex PCR assays for nearly all Ig/TCR targets in humans[48,106]. Despite the global standardization of the clonality assay in humans, the specificity of the TCR rearrangement assay, was found to be only 54.3%. In veterinary medicine, standard criteria for primers, conduction of the assay, and interpretation of results are lacking[49]. Similarly, this study suggested a high rate of false-positive results for the PARR assay in healthy cats. Based on this data and data available from human medicine, reclassification of cases based on results of PARR alone is not justified. Further studies investigating other methods for the diagnosis and differentiation of feline IBD from SCL are warranted. One possible approach could be the application of histology-guided mass spectrometry (HGMS). HGMS is a proprietary application of matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) that allows for the analysis of endogenous molecules directly in tissue sections with the assistance of histopathology annotation for the targeted analysis of cell subpopulations[218]. A study investigating the use of HGMS profiling to generate *in situ* molecular fingerprinting from formalin-fixed paraffin-embedded tissue sections to distinguish IBD from SCL in cats with chronic signs of gastrointestinal disease is current ongoing.

**Conclusion**

The current gold standards for the diagnosis of feline CE, histopathology, immunohistochemistry, and PCR-based clonality assays, frequently show results that are

111

considered "abnormal" in healthy, client-owned cats. Therefore, the development of new diagnostic modalities that reliably differentiate between healthy cats and cats with CE are warranted. Based on findings of this study, the reclassification of cases based on a single test alone is not justified in cats with CE.

## 6.2. Biomarker discovery by 2D DIGE based proteomics analysis

Limitations of the tests discussed above make the development of less invasive more reliable biomarkers for the diagnosis and differentiation of feline CE desirable. Several protein biomarker candidates were identified in the intestinal mucosa of cats with CE based on 2D DIGE proteomics. Inorganic pyrophosphatase has previously been described as a diagnostic and prognostic marker in patients with gastrointestinal[127,128] and other forms of cancer. Overexpression of Annexin A4 was previously found to be associated with tumor progression and chemo resistance in gastrointestinal neoplasia[132]. Cytosolic and mitochondrial malate dehydrogenases have been found to facilitate glycolysis *in vitro* in proliferating cancer cells including a canine lymphoma cell line[135-137]. Based on measurements of the protein spot intensity, these proteins were found to be differentially expressed in the small intestinal mucosa of cats with CE compared to healthy controls. In addition, these proteins were differentially expressed between cats with IBD and SCL and thus were identified as biomarker candidates for the diagnosis and differentiation of IBD and SCL in cats. However, many omics experiments suffer from a high rate of false-positive findings and, although they provide compound rations between different groups, these techniques generally do not provide absolute compound

112

quantities[52]. Therefore, all biomarker candidates have to undergo a verification using a different, quantitative or semi-quantitative method such as Western Blotting[53,60].

**Conclusion**

2D DIGE based proteomics revealed several protein biomarker candidates for the diagnosis and differentiation of feline IBD and SCL. While some of the proteins found in this study have previously been described as diagnostic and prognostic markers *in vivo* and *in vitro*, others do not have an immediate affiliation with a pathologic condition. Further studies to verify these findings are warranted.

**6.3. Biomarker discovery by characterization of the fecal microbiome and metabolome**

In recent years the significance of the intestinal microbiome and metabolome as an indicator as well as influential factor on the host health has become increasingly clear. In humans and dogs with chronic gastrointestinal disease, the fecal bacterial diversity is significantly decreased and associated with a worse prognosis[69,70,75,150]. Despite variations among different species, a patterns of microbial shifts characteristic of a dysbiotic state have been described: a reduction of the overall diversity of bacterial species (alpha diversity), a higher fluctuation rate over time, and a reduction of members of obligate anaerobic taxa in favor of facultative anaerobic taxa (e.g., members of the family *Enterobacteriaceae*)[73,147,150]. Similarly, to other species, cats with CE showed a significantly decreased alpha diversity. In addition, cats with CE tended to have a lower abundance of obligate anaerobic taxa (*Bacteroidetes*, *Firmicutes,* and *Actinobacteria*) while facultative anaerobic taxa (*Enterobacteriaceae* and *Streptococcaceae*) tended to be

increased. In humans and dogs, dysbiosis indices were previously developed to standardize microbiome profiling and characterize global changes of the intestinal microbiome in patients with CE[78,81,150]. Bacterial taxa significantly altered in dogs with CE were identified by untargeted 16S rRNA sequencing, verified by qPCR, and built into a mathematical model to find the highest discriminatory power[81]. Similar studies in cats with CE are currently underway to establish a dysbiosis index for feline CE.

Besides perturbations of the microbiome, alterations of the fecal metabolome have previously been described in humans and dogs with CE[79,87,183]. Metabolic compounds and pathways commonly affected by chronic intestinal inflammation are amino acids, bile acids, fatty acids, and metabolites within the tryptophan pathway such as indoles[79,183,185]. Cats with IBD or SCL showed similar alterations of the fecal metabolome to those observed in other species. Therefore, the metabolic consequences of intestinal inflammation appear to be similar between cats, dogs and humans. However, while changes in the fecal metabolome between healthy individuals and patients with CE are commonly robust, metabolic profiles between different forms of CE in people appear to be less pronounced between different gastrointestinal diseases, where only a few compounds hold differential power[185]. Similar observations were made in cats with CE. Marked differences in the fecal metabolome were found between healthy cats and cats with CE. In contrast, changes between cats with IBD and cats with SCL were less pronounced with only a few metabolites differently expressed between groups. It remains unclear whether alterations in the fecal metabolome are a cause or a

consequence of the disease and further studies to investigate disease pathogenesis are required.

**Conclusion**

The characterization of the fecal microbiome and metabolome in cats with CE revealed global metabolic changes and dysbiosis. Several bacterial taxa and metabolites were identified that hold discriminatory power to differentiate healthy cats from cats with CE and also between cats with IBD or SCL. Future studies using targeted assays such as quantitative PCRs and targeted mass spectrometry to verify these findings are required.

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143

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# APPENDIX A

## SUPPLEMENTAL INFORMATION FOR CHAPTER 2. RESULTS OF HISTOPATHOLOGY, IMMUNOHISTOCHEMISTRY AND MOLECULAR CLONALITY TESTING OF SMALL INTESTINAL BIOPSY SPECIMENS FROM CLINICALLY HEALTHY CLIENT-OWNED CATS

Table 6.1. Detailed summary of results of histopathology, immunohistochemistry, and molecular clonality testing of small intestinal biopsies from clinically healthy, client-owned cats.

| # | Age | Sex | Breed | Vomiting | Laboratory results | Histopathologic interpretation stomach | Mean WSAVA score (stomach) | Histopathologic interpretation duodenum | Mean WSAVA score (duodenum) | IHC | PARR | Integrated interpretation (based on H&E, IHC, and PARR) | Follow up period (days) | Outcome at last follow-up |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 13 | FS | Persian | occasionally hairballs ≤ 2x/months | Folate 33.8 µg/L | mod. LPG | 3.5 | mod. LPE | 3 | epitheliotropic infiltrate CD3+ | clonal in a polyclonal background | T-SCL, epitheliotropic | 869 | normal |
| 2 | 9 | FS | DSH | occasionally, ≤ 2x/months | Folate 22.4 µg/L | mild – mod. LPG | 0.5 | minimal - mild LPE | 1.5 | epitheliotropic infiltrate CD3+ | clonal in a polyclonal background | T-SCL, epitheliotropic | 837 | normal |
| 3 | 3 | MN | DSH | none | WNL | mild LPG | 1.5 | mild – mod. LPE | 2 | CD3+ | polyclonal | LPE | 821 | normal |
| 4 | 11 | FS | DSH | none | WNL | mild – mod. LPG | 2.5 | moderate LPE | 3 | epitheliotropic infiltrate CD3+ | clonal | T-SCL, epitheliotropic | 800 | normal |
| 5 | 10 | FS | DSH | none | WNL | mild LPG | 2.5 | minimal to mild LPE | 2.5 | CD3+ | polyclonal | LPE | 736 | normal |

Table 6.1. Continued. Detailed summary of results of histopathology, immunohistochemistry, and molecular clonality testing of small intestinal biopsies from clinically healthy, client-owned cats.

| # | Age | Sex | Breed | Vomiting | Laboratory results | Histopathologic interpretation stomach | Mean WSAVA score (stomach) | Histopathologic interpretation duodenum | Mean WSAVA score (duodenum) | IHC | PARR | Integrated interpretation (based on H&E, IHC, and PARR) | Follow up period (days) | Outcome at last follow-up |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 6 | 9 | FS | DSH | none | WNL | minimal LPG | 0.5 | mild – mod. LPE | 2 | epitheliotropic infiltrate CD3+ | pseudoclonal | uninterpretable | 734 | normal |
| 7 | 12 | FS | DLH | none | WNL | NA | NA | mild to moderate LPE | 3.5 | epitheliotropic infiltrate CD3+ | clonal in a polyclonal background | T-SCL, epitheliotropic | 718 | normal |
| 8 | 9 | FS | DSH | none | fPLI 15.6 µg/L | NA | NA | mod. LPE | 4.5 | epitheliotropic infiltrate CD3+ | clonal | T-SCL, epitheliotropic | 718 | normal |
| 9 | 11 | MN | Burmese | none | Folate 25.3 µg/L | mild LPG | 1 | mild LPE | 2 | epitheliotropic infiltrate CD3+ | clonal | T-SCL, epitheliotropic | 709 | normal |
| 10 | 18 | FS | DSH | occasionally, ≤ 2x/months | Folate 27.3 µg/L | mild LPG | 0.5 | SCL | 5 | epitheliotropic infiltrate CD3+ | clonal in a polyclonal background | T-SCL, epitheliotropic | 654 | euthanized due to GI signs |
| 11 | 9 | FS | Norwegian Forest Cat | none | Folate 65.5 µg/L | minimal to mild LPG | 1.5 | mild – mod. LPE | 2.5 | epitheliotropic + LP infiltrate CD3+ | clonal in a polyclonal background | emerging T-SCL, epitheliotropic | 647 | normal |
| 12 | 8 | FS | DSH | none | Folate 62.5 µg/L | minimal to mild LPG | 1.5 | mild LPE | 2.5 | epitheliotropic + LP infiltrate CD3+ | polyclonal | LPE | 641 | normal |

149

Table 6.1. Continued. Detailed summary of results of histopathology, immunohistochemistry, and molecular clonality testing of small intestinal biopsies from clinically healthy, client-owned cats.

| # | Age | Sex | Breed | Vomiting | Laboratory results | Histopathologic interpretation stomach | Mean WSAVA score (stomach) | Histopathologic interpretation duodenum | Mean WSAVA score (duodenum) | IHC | PARR | Integrated interpretation (based on H&E, IHC, and PARR) | Follow up period (days) | Outcome at last follow-up |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 13 | 8 | MN | DSH | none | WNL | mild to moderate LPG | 2.5 | mild LPE | 2 | epitheliotropic infiltrate CD3+ | polyclonal | LPE | 544 | on hypoallergenic diet due to vomiting |
| 14 | 13 | MN | DLH | none | WNL | mild - mod. LPG | 1 | mild -mod. LPE | 3.5 | epithelial + LP CD3+ | clonal | T-SCL, epitheliotropic | 501 | normal |
| 15 | 14 | FS | DSH | none | WNL | moderate LPG (focally extensive and nodular LPG) | 3.5 | mild LPE | 1.5 | epithelial + LP CD3+ | clonal | T-SCL, epitheliotropic | 493 | normal |
| 16 | 11 | MN | DLH | none | WNL | minimal LPG | 1 | minimal - mild LPE | 2.5 | CD3+ | clonal | T-SCL, epitheliotropic | 428 | normal |
| 17 | 8 | MN | DSH | none | WNL | minimal LPG | 0.5 | minimal – mild LPE | 2 | epitheliotropic infiltrate CD3+ | polyclonal | LPE | 351 | normal |
| 18 | 8 | MN | DSH | none | WNL | mild - moderate LPG | 3 | mild - mod. LPE | 1.5 | epitheliotropic infiltrate CD3+ | polyclonal | LPE | 342 | normal |

150

Table 6.1. Continued. Detailed summary of results of histopathology, immunohistochemistry, and molecular clonality testing of small intestinal biopsies from clinically healthy client-owned cats.

| # | Age | Sex | Breed | Vomiting | Laboratory results | Histopathologic interpretation stomach | Mean WSAVA score (stomach) | Histopathologic interpretation duodenum | Mean WSAVA score (duodenum) | IHC | PARR | Integrated interpretation (based on H&E, IHC, and PARR) | Follow up period (days) | Outcome at last follow-up |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 19 | 11 | FS | Siamese | occasionally, ≤ 2x/month | WNL | minimal LPG | 2.5 | SCL | 5.5 | epithelial + LP CD3+ | clonal | Small to intermediate lymphoma | 295 | euthanized due to GI signs |
| 20 | 9 | MN | Siamese | none | WNL | mild LPG | 1.5 | mod. LPE | 2.5 | epitheliotropic infiltrate CD3+ | clonal | T-SCL, epitheliotropic | 219 | normal |

Abbreviations: FS, female spayed; GI, gastrointestinal; IHC immunohistochemistry; LP, lamina propria; LPG, lymphocytic-plasmacytic gastritis; LPE, lymphocytic-plasmacytic enteritis; mod., moderate; MN, male neutered; PARR, PCR for antigen receptor rearrangement; SCL; small cell lymphoma; T-SCL, T cell small cell lymphoma; WNL, within normal limits; WSAVA, World Small Animal Veterinary Association; Reference interval for folate: 9.7-21.6 µg/L, Cut-off value for fPLI ≤3.5 µg/L.

151

Table 6.2. Histologic scores according to the diagnostic criteria by the World Small Animal Veterinary Association

| | Stomach | | | | | | | | | Duodenum | | | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | Morphological features | | | | Inflammation | | | | | Morphological features | | | | | | Inflammation | | | |
| Cat # | Surface epithelium | Gastric pit epithelium | Mucosal fibrosis | IEL | LP lymphocytes and plasma cells | LP eosinophils | LP neutrophils | LP macrophages | Gastric lymphoid follicular hyperplasia | Surface epithelium | Villus stunting | Crypt dilation/ distortion | Lacteal dilation | Mucosal fibrosis | IEL | LP lymphocytes and plasma cells | LP eosinophils | LP neutrophils | LP macrophages |
| 1 | 0 | 0 | 1 | 0 | 2-3 | 0 | 0 | 0 | 0 | 0 | 0 | 0-1 | 0 | 0-1 | 0-1 | 1-2 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0-1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0-1 | 0 | 0-1 | 0-1 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0-1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0-1 | 0 | 0 | 0-1 | 1 | 0 | 0 | 0 |
| 4 | 0 | 0 | 0-1 | 0 | 0-1 | 0 | 0 | 0 | 1 | 0 | 0 | 0-1 | 0 | 0 | 0-1 | 2 | 0 | 0 | 0 |
| 5 | 0 | 0 | 1-2 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0-1 | 0 | 0-1 | 0-1 | 1 | 0 | 0 | 0 |
| 6 | 0 | 0 | 0 | 0 | 0-1 | 0 | 0 | 0 | 0 | 0 | 0 | 0-1 | 0 | 0 | 0-1 | 1 | 0 | 0 | 0 |
| 7 | NA | NA | NA | NA | NA | NA | NA | NA | NA | 0 | 0 | 0-1 | 0 | 0-1 | 1 | 1-2 | 0 | 0 | 0 |
| 8 | NA | NA | NA | NA | NA | NA | NA | NA | NA | 0 | 0 | 1 | 0-1 | 0 | 1 | 2 | 0 | 0 | 0 |
| 9 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0-1 | 0 | 0 | 0-1 | 1 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 | 0 | 0-1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 3 | 0 | 0 | 0 |
| 11 | 0 | 0 | 0-1 | 0 | 0-1 | 0 | 0 | 0 | 0 | 0 | 0 | 0-1 | 0 | 0-1 | 0-1 | 1 | 0 | 0 | 0 |
| 12 | 0 | 0 | 0-1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0-1 | 0 | 0-1 | 0-1 | 1 | 0 | 0 | 0 |
| 13 | 0 | 0 | 0-1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0-1 | 0 | 0 | 0-1 | 1 | 0 | 0 | 0 |

Table 6.2. Continued. Histologic scores according to the diagnostic criteria by the World Small Animal Veterinary Association

| | Stomach | | | | | | | | | Duodenum | | | | | | | | | |
| | Morphologic al features | | | Inflammation | | | | | | Morphological features | | | | | Inflammation | | | | |
| Cat # | Surface epithelium | Gastric pit epithelium | Mucosal fibrosis | IEL | LP lymphocytes and plasma cells | LP eosinophils | LP neutrophils | LP macrophages | Gastric lymphoid follicular hyperplasia | Surface epithelium | Villus stunting | Crypt dilation/ distortion | Lacteal dilation | Mucosal fibrosis | IEL | LP lymphocytes and plasma cells | LP eosinophils | LP neutrophils | LP macrophages |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 14 | 0 | 0 | 0-1 | 0 | 0-1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0-1 | 0 | 0-1 | 1-2 | 0 | 0 | 0 |
| 15 | 0 | 0 | 0-1 | 0 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0-1 | 1 | 0 | 0 | 0 |
| 16 | 0 | 0 | 0-1 | 0 | 0-1 | 0 | 0 | 0 | 0 | 0 | 0 | 0-1 | 0 | 0-1 | 0-1 | 1 | 0 | 0 | 0 |
| 17 | 0 | 0 | 0 | 0 | 0-1 | 0 | 0 | 0 | 0 | 0 | 0 | 0-1 | 0 | 0 | 0-1 | 1 | 0 | 0 | 0 |
| 18 | 0 | 0 | 0-1 | 0 | 1-2 | 0 | 0 | 0 | 1 | 0 | 0 | 0-1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 19 | 0 | 0 | 0-1 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0-1 | 0-1 | 0 | 0 | 2-3 | 2 | 0 | 0 | 0 |
| 20 | 0 | 0 | 0-1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0-1 | 0 | 0 | 0-1 | 1-2 | 0 | 0 | 0 |
| Median | 0 | 0 | 0.5 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0.5 | 0 | 0 | 0.5 | 1 | 0 | 0 | 0 |
| Range | 0 | 0 | 0-1 | 0 | 0-3 | 0 | 0 | 0 | 0 | 0 | 0-1 | 0-1 | 0-1 | 0-1 | 0-3 | 0.5 - 3 | 0 | 0 | 0 |

Abbreviations: 0, absence of changes; 1, mild changes; 2 moderate changes; 3, severe changes; IEL, intraepithelial lymphocytes; LP, lamina propria; NA, not assessed.

APPENDIX B

SUPPLEMENTAL INFORMATION FOR CHAPTER 4. CHARACTERIZATION OF THE FECAL MICROBIOME IN

CATS WITH INFLAMMATORY BOWEL DISEASE OR ALIMENTARY LYMPHOMA

Table 6.3. Summary of alpha diversity indices at a depth of 43,660 sequences per sample comparing healthy cats and cats with CE (FCE) with subgroups inflammatory bowel disease (IBD) and small cell lymphoma (SCL).

| Index | Healthy | FCE | | Subgroup IBD | Subgroup SCL | | Dunn's post hoc p value | | |
| | | | | | | Kruskal Wallis | | | IBD/ |
| | Median (range) | Median (range) | P value | Median (range) | Median (range) | ANOVA P value | H/IBD | H/SCL | SCL |
|---|---|---|---|---|---|---|---|---|---|
| **OTUs** | 253.5 (167 – 359) | 232 (98 – 359) | 0.003 | 232 (119 – 359) | 228 (98 – 265) | 0.015 | 0.120 | 0.035 | >0.999 |
| **Shannon** | 7.3 (6.5 – 8.0) | 7.0 (5.1 – 7.5) | 0.008 | 7.0 (5.5 – 7.5) | 7.0 (5.1 – 7.5) | 0.030 | 0.100 | 0.112 | >0.999 |
| **Faith PD** | 9.5 (6.9 – 11.8) | 8.7 (5.6 – 10.7) | 0.019 | 9.0 (6.1 – 9.6) | 8.3 (5.6 – 10.7) | 0.049 | 0.500 | 0.061 | >0.999 |

Abbreviations: FCE Feline chronic enteropathy, OTUs observed operational taxonomic units, STD Standard deviation, PD Phylogenetic Index, H Healthy cats

154

Table 6.4. Taxa found to be significantly different (p value) between healthy cats, cats with idiopathic inflammatory bowel disease (IBD), and cats small cell lymphoma (SCL) before correction for false discovery (q value).

| Bacterial Group | Healthy | | IBD | | SCL | | Healthy vs IBD vs SCL[1] | | Healthy vs IBD[2] | | Healthy vs SCL[2] | | IBD vs SCL[2] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Median | Range | Median | Range | Median | Range | P value | Q value | P value | Q value | P value | Q value | P value | Q value |
| **Class** | | | | | | | | | | | | | | |
| Actinobacteria | 1.2 | 0-36.8 | 0.4 | 0.2-38.9 | 0.2 | 0.1-18.4 | 0.034 | 0.360 | 1.000 | 1.000 | 0.028 | 0.309 | 0.279 | 1.000 |
| Gammaproteobacteria | 0.7 | 0-32.6 | 0.5 | 0-39.5 | 1.8 | 0.2-37.8 | 0.073 | 0.360 | 1.000 | 1.000 | 0.101 | 0.554 | 0.155 | 1.000 |
| **Order** | | | | | | | | | | | | | | |
| Bifidobacteriales | 1.2 | 0-36.8 | 0.4 | 0.2-38.9 | 0.2 | 0.1-18.4 | 0.030 | 0.193 | 1.000 | 1.000 | 0.024 | 0.156 | 0.361 | 1.000 |
| Turicibacterales | 0 | 0-23.7 | 0 | 0-5.8 | 0 | 0-0.1 | 0.074 | 0.320 | 0.784 | 1.000 | 0.081 | 0.350 | 1.000 | 1.000 |
| Enterobacteriales | 0.1 | 0-32.6 | 0.2 | 0-39.4 | 1 | 0-37.8 | 0.019 | 0.193 | 0.564 | 1.000 | 0.018 | 0.156 | 0.767 | 1.000 |
| **Family** | | | | | | | | | | | | | | |
| Bifidobacteriaceae | 1.2 | 0-36.8 | 0.4 | 0.2-38.9 | 0.2 | 0.1-18.4 | 0.030 | 0.166 | 1.000 | 1.000 | 0.024 | 0.237 | 0.361 | 1.000 |
| Prevotellaceae | 1.1 | 0-21.7 | 0.6 | 0.2-18.8 | 0.1 | 0-22.5 | 0.037 | 0.170 | 1.000 | 1.000 | 0.031 | 0.237 | 0.275 | 1.000 |
| Odoribacteraceae | 0 | 0-2.8 | 0 | 0-1.3 | 0.8 | 0-1.4 | 0.009 | 0.130 | 1.000 | 1.000 | 0.009 | 0.237 | 0.055 | 1.000 |
| Paraprevotellaceae | 0 | 0-18.5 | 0 | 0-0.3 | 0 | 0-5.6 | 0.009 | 0.130 | 0.061 | 0.493 | 0.034 | 0.237 | 1.000 | 1.000 |
| Streptococcaceae | 0 | 0-4.1 | 0.1 | 0-4.3 | 0.1 | 0-61.4 | 0.022 | 0.155 | 0.070 | 0.493 | 0.103 | 0.481 | 1.000 | 1.000 |
| Ruminococcaceae | 8 | 1.2-18.4 | 5.8 | 0.3-9.8 | 5.6 | 0.2-17.3 | 0.053 | 0.194 | 0.065 | 0.493 | 0.540 | 1.000 | 1.000 | 1.000 |
| Enterobacteriaceae | 0.1 | 0-32.6 | 0.2 | 0-39.4 | 1 | 0-37.8 | 0.019 | 0.155 | 0.738 | 1.000 | 0.495 | 1.000 | 1.000 | 1.000 |

Table 6.4. Continued. Taxa found to be significantly different (p value) between healthy cats, cats with idiopathic inflammatory bowel disease (IBD), and cats small cell lymphoma (SCL) before correction for false discovery (q value). Numbers represent relative percentages.

| Bacterial Group | Healthy | | IBD | | SCL | | Healthy vs IBD vs SCL[1] | | Healthy vs IBD[2] | | Healthy vs SCL[2] | | IBD vs SCL[2] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Median | Range | Median | Range | Median | Range | P value | Q value | P value | Q value | P value | Q value | P value | Q value |
| **Genus** | | | | | | | | | | | | | | |
| Bifidobacterium | 1.2 | 0-36.8 | 0.4 | 0.2-38.9 | 0.2 | 0.1-18.4 | 0.030 | 0.238 | 1.000 | 1.000 | 0.024 | 0.494 | 0.361 | 1.000 |
| Undetermined genus, Coriobacteriaceae | 0.1 | 0-16.4 | 0.4 | 0-11.3 | 0.1 | 0-7.2 | 0.026 | 0.238 | 0.453 | 1.000 | 0.199 | 0.735 | 0.022 | 1.000 |
| Prevotella | 1.1 | 0-21.7 | 0.6 | 0.2-18.8 | 0.1 | 0-22.5 | 0.037 | 0.250 | 1.000 | 1.000 | 0.031 | 0.494 | 0.275 | 1.000 |
| Odoribacter | 0 | 0-2.8 | 0 | 0-1.3 | 0.3 | 0-1.4 | 0.051 | 0.281 | 1.000 | 1.000 | 0.047 | 0.566 | 0.268 | 1.000 |
| Streptococcus | 0 | 0-4.1 | 0.1 | 0-4.3 | 0.1 | 0-61.4 | 0.015 | 0.238 | 0.054 | 0.646 | 0.073 | 0.634 | 1.000 | 1.000 |
| Peptococcus | 0.4 | 0-3 | 0.4 | 0-2.8 | 0.3 | 0-2 | 0.957 | 0.957 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| Undetermined genus, Peptostreptococcaceae | 0.3 | 0-16.6 | 0.1 | 0-1 | 0.1 | 0-5.9 | 0.121 | 0.353 | 0.284 | 1.000 | 0.333 | 1.000 | 1.000 | 1.000 |
| Undetermined genus, Ruminococcaceae | 0.1 | 0-0.9 | 0 | 0-0.4 | 0 | 0-0.5 | 0.013 | 0.238 | 0.036 | 0.646 | 0.093 | 0.634 | 1.000 | 1.000 |
| Oscillospira | 1.1 | 0.1-3.5 | 0.4 | 0-1.5 | 0.9 | 0-2.2 | 0.025 | 0.238 | 0.021 | 0.646 | 0.816 | 1.000 | 0.526 | 1.000 |
| Undetermined genus, Enterobacteriaceae | 0.1 | 0-32.6 | 0.2 | 0-39.4 | 1 | 0-37.8 | 0.016 | 0.238 | 0.507 | 1.000 | 0.015 | 0.494 | 0.782 | 1.000 |
| **Species** | | | | | | | | | | | | | | |
| Undetermined species, Bifidobacterium | 1.1 | 0-35.2 | 0.4 | 0-38.6 | 0.1 | 0-11.5 | 0.047 | 0.259 | 0.249 | 0.962 | 0.063 | 0.530 | 1.000 | 1.000 |
| Undetermined species, Coriobacteriaceae | 0.1 | 0-16.4 | 0.4 | 0-11.3 | 0.1 | 0-7.2 | 0.026 | 0.217 | 0.453 | 1.000 | 0.199 | 0.734 | 0.022 | 1.000 |
| Undetermined species, Bacteroides | 0.1 | 0-2.3 | 0 | 0-0.8 | 0 | 0-0.5 | 0.009 | 0.188 | 0.340 | 1.000 | 0.010 | 0.451 | 0.860 | 1.000 |
| Bacteroides plebeius | 1.2 | 0-14.5 | 0.1 | 0-10.9 | 0.1 | 0-4.8 | 0.014 | 0.188 | 0.141 | 0.642 | 0.030 | 0.464 | 1.000 | 1.000 |
| Prevotella copri | 1.1 | 0-21.7 | 0.6 | 0.2-18.8 | 0.1 | 0-22.5 | 0.046 | 0.259 | 1.000 | 1.000 | 0.041 | 0.464 | 0.279 | 1.000 |

156

Table 6.4. Continued. Taxa found to be significantly different (p value) between healthy cats, cats with idiopathic inflammatory bowel disease (IBD), and cats small cell lymphoma (SCL) before correction for false discovery (q value). Numbers represent relative percentages.

| Bacterial Group | Healthy | | IBD | | SCL | | Healthy vs IBD vs SCL[1] | | Healthy vs IBD[2] | | Healthy vs SCL[2] | | IBD vs SCL[2] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Median | Range | Median | Range | Median | Range | P value | Q value | P value | Q value | P value | Q value | P value | Q value |
| **Species** | | | | | | | | | | | | | | |
| Undetermined species, Odoribacter | 0 | 0-2.8 | 0 | 0-1.3 | 0.3 | 0-1.4 | 0.051 | 0.259 | 1.000 | 1.000 | 0.047 | 0.464 | 0.268 | 1.000 |
| Undetermined species, Streptococcus | 0 | 0-4.1 | 0.1 | 0-4.3 | 0.1 | 0-61.4 | 0.033 | 0.243 | 0.055 | 0.620 | 0.267 | 0.875 | 1.000 | 1.000 |
| Undetermined species, Turicibacter | 0 | 0-23.7 | 0 | 0-5.8 | 0 | 0-0.1 | 0.074 | 0.259 | 0.784 | 1.000 | 0.081 | 0.530 | 1.000 | 1.000 |
| Undetermined species, Clostridiales | 1 | 0-4.7 | 0.5 | 0-5.1 | 1.3 | 0-5.1 | 0.056 | 0.259 | 0.049 | 0.620 | 1.000 | 1.000 | 0.405 | 1.000 |
| Undetermined species,Clostridium | 0.2 | 0-10.8 | 0.9 | 0.1-4.2 | 0.8 | 0.1-20.3 | 0.009 | 0.188 | 0.046 | 0.620 | 0.045 | 0.464 | 1.000 | 1.000 |
| Undetermined species,Ruminococcaceae | 0.1 | 0-0.9 | 0 | 0-0.4 | 0 | 0-0.5 | 0.013 | 0.188 | 0.036 | 0.620 | 0.093 | 0.546 | 1.000 | 1.000 |
| Undetermined species, Oscillospira | 1.1 | 0.1-3.5 | 0.4 | 0-1.5 | 0.9 | 0-2.2 | 0.025 | 0.217 | 0.021 | 0.620 | 0.816 | 1.000 | 0.526 | 1.000 |
| Undetermined species, Enterobacteriaceae | 0.1 | 0-32.6 | 0.2 | 0-39.4 | 1 | 0-37.8 | 0.016 | 0.188 | 0.507 | 1.000 | 0.015 | 0.451 | 0.782 | 1.000 |

Numbers represent relative percentages. Legend: [1] Kruskal Wallis ANOVA, [2] Dunn's post hoc test

APPENDIX C

SUPPLEMENTAL INFORMATION FOR CHAPTER 5. UNTARGETED

METABOLOMIC ANALYSIS IN CATS WITH NATURALLY OCCURRING

INFLAMMATORY BOWEL DISEASE AND ALIMENTARY SMALL CELL

LYMPHOMA


Table 6.5. Metabolites identified in feces healthy control cats (healthy) and cats with chronic enteropathy (FCE). The data is mean centered.

The data is available as a supplemental file.

Table 6.6. Metabolites identified in feces of healthy control cats (healthy) cats with inflammatory bowel disease (IBD) and cats with alimentary small cell lymphoma (SCL). The data is mean centered. Legend: [1] Kruskal Wallis ANOVA, [2] Dunn's post hoc test

The data is available as a supplemental file.