DEVELOPMENT AND ANALYTICAL VALIDATION OF AN ENZYME-LINKED IMMUNOSORBANT ASSAY (ELISA) FOR THE MEASUREMENT OF FELINE ALPHA$_1$-PROTEINASE INHIBITOR (fα_1-PI) IN SERUM AND FECES AND THE EVALUATION OF FECAL fα_1-PI CONCENTRATIONS IN CATS WITH IDIOPATHIC INFLAMMATORY BOWEL DISEASE OR GASTROINTESTINAL NEOPLASIA

A Thesis

by

KATHRIN FETZ BURKE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2012

Major Subject: Biomedical Sciences

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Approved by:

Chair of Committee,	Jörg M. Steiner
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	John R. August
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ABSTRACT

Development and Analytical Validation of an Enzyme-Linked Immunosorbent Assay (ELISA) for the Measurement of Feline Alpha$_1$-Proteinase Inhibitor (fα_1-PI) in Serum and Feces and the Evaluation of Fecal fα_1-PI Concentrations in Cats with Idiopathic Inflammatory Bowel Disease or Gastrointestinal Neoplasia. (August 2012)

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Chair of Advisory Committee: Dr. Jörg M. Steiner

Alpha$_1$-proteinase inhibitor (α_1-PI) has been shown to be a useful marker of gastrointestinal protein loss in some species. The objectives of this study were, first, to develop and analytically validate an ELISA for the measurement of α_1-PI in feces and serum from cats, and, second, to evaluate fecal α_1-PI concentrations in healthy cats and cats with chronic gastrointestinal disease.

The lower detection limits of the ELISA were 0.02 g/L for serum and 0.04 µg/g for feces. The observed-to-expected (O/E) ratios for serial dilutions of serum and fecal samples ranged from 100.0 to 129.7% (mean ±SD: 112.2 ±9.9%) and 103.5 to 141.6% (115.6 ±12.8%), respectively. The O/E ratios for samples spiked with seven known concentrations of α_1-PI ranged from 82.3 to 107.8% (94.7 ±7.6%) for serum and 78.5 to 148.7% (96.8 ±18.2%) for feces. The coefficients of variation for intra-assay and inter-assay variability were <7.9% and <12.1% for serum, and 5.3%, 11.8%, and 14.2% and

7.7%, 10.2%, and 20.4% for feces, respectively. Reference intervals were 0.6 to 1.4 g/L for serum and up to 1.6 µg/g for feces. We conclude that this ELISA is sufficiently linear, accurate, precise, and reproducible.

For the clinical evaluation, twenty cats with clinical signs of chronic gastrointestinal disease and 20 healthy control cats were enrolled. The diseased cats were grouped into two groups: mild to moderate idiopathic inflammatory bowel disease (IBD) (Group A; n=8) and severe IBD or neoplastic disease (Group B; n=12), based on histopathology results of endoscopic biopsies. Fecal α_1-PI concentrations and serum concentrations of total protein, albumin, globulin, cobalamin, folate, pancreatic lipase immunoreactivity, and trypsin-like immunoreactivity were determined. Nineteen of the 20 diseased cats had increased fecal α_1-PI concentrations, ranging from 1.9 to 233.6 µg/g (normal range: ≤1.6 µg/g). Fecal α_1-PI concentrations were statistically significantly different between healthy cats and cats of Group A (median: 3.9 µg/g, range: 1.3 to 9.2 µg/g, $P<0.001$) or cats of Group B (median: 20.6 µg/g, 4.3 to 233.6 µg/g; $P<0.001$), and also between cats of Groups A and B ($P<0.01$). Hypoalbuminemia, hypoproteinemia, and hypocobalaminemia were detected in 88%, 83%, and 56% of the diseased cats, respectively. Our study suggests that increased fecal α_1-PI concentrations in association with hypoalbuminemia may be a common finding in cats with IBD or GI neoplasia. Furthermore, α_1-PI concentrations appear to be higher in cats with severe IBD or confirmed GI neoplasia when compared to cats with mild to moderate IBD.

DEDICATION

To Adam and our family

ACKNOWLEDGEMENTS

I would especially like to thank my committee chair Dr. Jörg Steiner and my committee member Dr. Jan Suchodolski for their guidance, patience, support, and most of all for their exceptional friendship throughout my time at the Gastrointestinal Laboratory. I would also like to thank Dr. Craig Ruaux, Dr. David Williams, and Dr. John Broussard for their help with this project. Further, I would like to thank my committee member Dr. John August for his support with this thesis. Many thanks also to the entire GI lab team for all their help and friendship over the past years. Finally, I would like to thank my husband Adam for his love, support, and encouragement.

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CHAPTER I

INTRODUCTION

ALPHA$_1$-PROTEINASE INHIBITOR

Alpha$_1$-proteinase inhibitor (α_1-PI) is a primarily hepatocellularly-produced glycoprotein that is present in various biological fluids, tissues, and cells, which is especially abundant in plasma. Alpha$_1$- PI is recognized as the prototype of the family of serine proteinase inhibitors (serpins), which represents the third largest group of plasma proteins after albumin and immunoglobulins (Hibbetts et al., 1999; Travis and Salvesen, 1983).

The main function of α_1-PI within the body consists of the regulation of various serine proteinases, in order to prevent excessive proteolysis in vivo. Alpha$_1$-PI was first identified in human sera by Schultze et al. (1955) and was named α_1-3.5-glycoprotein, owing to its migration within the α_1-globulin region during alkaline electrophoresis. In the same year, Jacobsson (1955) discovered that this protein appears to mainly inhibit trypsin, a serine proteinase. Therefore, the protein was renamed α_1-antitrypsin a few years later (Schultze et al., 1962).

This thesis follows the style of *The Veterinary Journal*.

However, other studies found that this protein shows the strongest inhibitory activity towards neutrophil elastase, but that it also regulates many other serine proteinases, including chymotrypsin, trypsin, and thrombin (Pannell et al., 1974). In order to reflect this broader spectrum of inhibitory activity terminologically, α_1-antitrypsin was re-named α_1-PI in the veterinary literature (Pannell et al., 1974). However, the name α_1-antitrypsin continues to be used more commonly in the human literature.

The terms "protease" and "proteinase" are often used interchangeably in the literature, which is however incorrect. Protease is a term that encompasses endopeptidases or exopeptidases, while proteinases only encompass endopeptidases. Endopeptidases break peptide bonds of nonterminal amino acids, in contrast to exopeptidases, which break peptide bonds of terminal amino acids. Serine proteinases, such as neutrophil elastase or trypsin, are all endopeptidases, and inhibitors of these proteinases, such as α_1-PI, are therefore correctly termed serine proteinase inhibitors (McDonald, 1985).

Alpha$_1$-PI has been identified and purified in many different species, including humans (Laurell et al., 1975), rhesus monkeys (Berninger and Mathis, 1976), horses (Patterson et al., 1991), and dogs (Melgarejo et al., 1996). In a previous study, we have purified and partially characterized α_1-PI in cats (Fetz et al., 2004).

In humans, α_1-PI has been shown to mainly inhibit the serine proteinase neutrophil elastase. The importance of this function became particularly evident when Laurell and Eriksson (1963) first described a state of α_1-PI deficiency in humans (this

disorder is still known as α_1-antitrypsin deficiency). Patients with this condition develop progressive lung emphysema due to insufficient inhibition of neutrophil elastase, which subsequently degrades elastin in the alveoli. Alpha$_1$-antitrypsin deficiency is now known to be one of the most frequent congenital disorders in humans (Graziadei et al., 1998). Alpha$_1$-antitrypsin deficiency has also been associated with chronic liver disease in humans, which is caused by accumulation of abnormal α_1-PI within hepatocytes (Nemeth, 1994). Further, α_1-PI has been shown to be an acute phase protein in humans as well as some other species, such as rabbits and rats, the plasma concentration of which may increase three- to four-fold during acute inflammation in humans (Alper et al., 1980; Koj and Regoeczi, 1978; Koj et al., 1978).

FECAL α_1-PI AS A MARKER OF GASTROINTESTINAL PROTEIN LOSS

In humans and dogs, various chronic gastroenteropathies that produce inflammation, vascular congestion, lymphatic occlusion, or bleeding have shown to be associated with transmucosal loss of plasma proteins, in particular albumin. Traditionally, gastrointestinal protein loss has been diagnosed by the measurement of radioactive chromium (^{51}Cr) in feces (Waldmann, 1961). For this method, ^{51}Cr is injected intravenously, which then binds to endogenous albumin. In case of transmucosal protein loss, the ^{51}Cr-labeled albumin is lost into the intestinal lumen. While albumin is degraded by proteolytic enzymes, the radioactivity of ^{51}Cr can still be detected in the fecal matter. This test is highly sensitive, but has important disadvantages. Most

importantly this test exposes both patients and personnel to radioactivity. Also, fecal matter has to be collected over a period of five days.

Because of its unique properties as a proteinase inhibitor, the measurement of α_1-proteinase inhibitor in feces was found to be a useful alternative method for the detection of gastrointestinal protein loss in humans and dogs (Melgarejo, 1999; Karbach, 1982). As α_1-PI has a similar molecular mass and charge to serum albumin, plasma α_1-PI tends to be lost into the gastrointestinal lumen whenever albumin leaks into the lumen as a result of gastrointestinal disease. But unlike albumin, α_1-PI is a proteinase inhibitor and is thus able to withstand degradation by digestive and bacterial proteases in the gastrointestinal lumen. Alpha$_1$-PI remains essentially intact in the gastrointestinal lumen and can therefore be quantified in feces by use of immunoassays.

An immunoassay, more specifically an ELISA, for the measurement of α_1-PI concentrations in fecal matter has been developed and analytically validated for use in dogs (Melgarejo et al., 1998), and has been shown to be clinically useful for the detection of protein loss in dogs with chronic GI disease (Murphy et al., 2003). Fecal α_1-PI concentration also may be useful in the detection of gastrointestinal protein loss in cats. However, immunoassays for α_1-PI are highly species-specific. In a previous study, we have purified α_1-PI from serum of cats and developed a species-specific radioimmunoassay for the detection of α_1-PI in serum of cats (Fetz et al., 2004); however, further studies showed this assay not to be useful for accurate quantification of α_1-PI in fecal matter of cats (unpublished data). Therefore, a need to develop and

analytically validate an immunoassay that allows for the measurement of α_1-PI in fecal samples from cats remained.

GASTROENTEROPATHIES WITH TRANSMUCOSAL PROTEIN LOSS

Abnormal loss of plasma proteins through the GI mucosa is also referred to as protein-losing enteropathy (PLE). Clinically, PLE is often identified in patients with hypoalbuminemia when transmucosal loss exceeds hepatic synthesis of albumin.

The exact mechanisms leading to transmucosal protein loss during GI disease are not clear. In human medicine, PLE can be classified according to the main pathologic changes leading to protein loss through the GI mucosa as erosive GI disorders, non-erosive GI disorders, and disorders involving increased central venous pressure or mesenteric lymphatic obstruction (Umar and DiBaise, 2010). In healthy individuals only small amounts of proteins are lost through the gut mucosa. According to a study by Schmidt et al. (1995), daily plasma protein loss into the GI lumen accounts for only 1 to 2% of the total plasma protein pool. In contrast, transmucosal protein loss in patients with PLE may reach up to 60% of the total albumin pool (Umar and DiBaise, 2010). PLE can be associated with a wide spectrum of disease conditions in humans, including Crohn's disease, gastric and intestinal neoplasia, systemic lupus erythematosus, or cardiac disease. In dogs, PLE has been mostly associated with idiopathic inflammatory bowel disease (IBD), intestinal lymphoma, and intestinal lymphangiectasia. In cats, there has been little mention of gastroenteropathies associated with protein loss. A few studies have reported hypoalbuminemia in cats with IBD (Baez et al., 1999; Bailey et al., 2010;

Jergens et al., 1992). Other authors have stated that PLE occurs less frequently in cats than in dogs (Peterson and Willard, 2003). However, supportive data for this hypothesis are lacking.

For the diagnosis of PLE, other disorders that have the potential to cause hypoalbuminemia, such as protein-losing nephropathy, liver insufficiency, or cutaneous losses have to be excluded first. The next step will involve the confirmation of protein loss through the GI tract. As mentioned above, the measurement of α_1-PI in feces has been shown to be suitable marker for GI protein loss in dogs, while studies in cats are not available to date.

HYPOTHESIS AND SPECIFIC OBJECTIVES

The hypotheses of this study are 1) that an enzyme-linked immunoassay can be used to measure α_1-PI inhibitor in serum and feces of cats and 2) that fecal α_1-PI concentrations are higher in cats with chronic GI disease compared to healthy cats.

The objectives of this study are 1) to develop and analytically validate an enzyme-linked immunoassay for the measurement of α_1-PI in serum and feces of cats and 2) to evaluate fecal α_1-PI concentrations in healthy cats and cats with idiopathic inflammatory bowel disease or gastrointestinal neoplasia.

CHAPTER II

DEVELOPMENT AND ANALYTICAL VALIDATION OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE MEASUREMENT OF ALPHA$_1$-PROTEINASE INHIBITOR IN SERUM AND FECES FROM CATS*

OVERVIEW

The objective of this study was to develop and analytically validate an ELISA for the measurement of alpha$_1$-proteinase inhibitor (α_1-PI) in feces and serum from cats. Lower detection limit, linearity, accuracy, precision, reproducibility, and reference intervals were determined. The lower detection limits were 0.02 g/L for serum and 0.04 µg/g for feces. The observed-to-expected (O/E) ratios for serial dilutions of serum and fecal samples ranged from 100.0 to 129.7% (mean ±SD: 112.2 ±9.9%) and 103.5 to 141.6% (115.6 ±12.8%), respectively. The O/E ratios for samples spiked with seven known concentrations of α_1-PI ranged from 82.3 to 107.8% (94.7 ±7.6%) for serum, and 78.5 to 148.7% (96.8 ±18.2%) for feces. The coefficients of variation for intra-assay and inter-assay variability were <7.9% and <12.1% for serum, and 5.3%, 11.8%, 14.2%, and 7.7%, 10.2%, 20.4% for feces, respectively. Reference intervals were 0.6 to 1.4 g/L for serum and up to 1.6 µg/g for feces. We conclude that this ELISA is sufficiently linear, accurate, precise, and reproducible for clinical evaluation.

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INTRODUCTION

Alpha$_1$-Proteinase inhibitor (α_1-PI) is a predominantly liver-produced glycoprotein, which protects tissues against excessive proteolysis by serine proteinases (Graziadei et al., 1998). Originally α_1-PI was thought to predominantly inhibit trypsin, and was therefore named α_1-antitrypsin (Schultze et al., 1962). Later it was found that many other serine proteinases are also regulated by this proteinase inhibitor, including neutrophil elastase, chymotrypsin, trypsin, and thrombin (Pannell et al., 1974). In order to reflect this broader spectrum of inhibitory activity terminologically, α_1-antitrypsin was named α_1-PI (Travis, 1986), however its original name is still more commonly used in the human literature. The important physiological role of α_1-PI in the body became particularly evident when Laurell and Eriksson (1963) first described a state of deficiency of α_1-antitrypsin in humans. Patients with this condition develop progressive lung emphysema due to insufficient inhibition of neutrophil elastase, which subsequently degrades elastin in the alveoli. Alpha$_1$-antitrypsin deficiency also has been associated with chronic liver disease in humans, which is likely related to accumulation of abnormal inhibitor protein within hepatocytes (Nemeth, 1994). Alpha$_1$-antitrypsin deficiency is now known to be one of the most frequent congenital disorders in humans (Graziadei et al., 1998). Mellor et al. (2006) have described a case of α_1-PI deficiency and concurrent *Bartonella* infection in association with panniculitis, polyarthritis, and meningitis in a Cavalier King Charles Spaniel. To the authors' knowledge, studies on abnormal or deficient α_1-PI or associated syndromes have not been reported in cats.

Owing to its property of inhibiting proteinases, α_1-PI escapes degradation in the gastrointestinal lumen, and its measurement in feces has been shown to be useful as a marker for gastrointestinal protein loss in both humans and dogs (Murphy et al., 2003; Karbach, 1983). In normal individuals, only minimal amounts of α_1-PI are present in the gastrointestinal lumen. As α_1-PI has a similar molecular mass and charge to albumin, α_1-PI tends to be lost into the gastrointestinal lumen whenever albumin leaks into the lumen as a result of increased mucosal leakage in disease states (Williams, 1996). As α_1-PI remains essentially intact in the gastrointestinal lumen, it can be used as a marker of transmucosal protein loss (Williams, 1996). An ELISA for the measurement of α_1-PI concentrations in fecal matter was developed and validated for dogs (Melgarejo et al., 1998), and shown to be useful in the detection of protein loss in dogs with chronic gastrointestinal disease (Murphy et al., 2003). Fecal α_1-PI concentration may also prove useful in the detection of gastrointestinal protein loss in cats. However, immunoassays for α_1-PI are species-specific. In a previous study, our group has purified feline α_1-PI (fα_1-PI) from serum of cats (Fetz et al., 2004). We further used radial immunodiffusion to evaluate cross-immunoreactivity of fα_1-PI antigen and antiserum of cats, dogs, and humans. In that study we were unable to demonstrate any immunologic cross-reactivity between these species (unpublished data, 2002). Thus, our group developed a species-specific radioimmunoassay for the detection of fα_1-PI in serum from cats (Fetz et al., 2004), but further studies showed that this assay was not useful to accurately quantify fα_1-PI in fecal matter of cats (unpublished data, 2004). Therefore, the aim of the present

study was to develop and analytically validate an immunoassay, which could be utilized for measurement of fα_1-PI in serum as well as fecal samples from cats.

MATERIALS AND METHODS

Purification of α_1-PI from feline serum. Feline α_1-PI was isolated from pooled feline serum samples using ammonium sulfate precipitation, anion-exchange, size-exclusion, ceramic hydroxyapatite, and hydrophobic interaction chromatography, as previously described (Fetz et al., 2004).

Production, purification, and biotinylation of antibodies. Polyclonal antibodies against fα_1-PI were raised in two New Zealand White rabbits by repeated inoculation with 150 μg of pure fα_1-PI emulsified in complete and incomplete Freund's adjuvant using a commercial antibody production service (Lampire Biological Laboratories, Pipersville, Pennsylvania). The rabbit anti-fα_1-PI antibodies were purified by affinity chromatography, using purified fα_1-PI coupled to N-hydroxysuccinamide-activated Sepharose (Amersham Biosciences Corp., Uppsala, Sweden). Purified antibodies from the first rabbit were used as capture antibodies, while purified antibodies from the second rabbit were biotinylated using a commercially available kit (Pierce, Rockford, Illinois), and used as reporter antibodies.

Collection of fecal and serum samples. Blood was collected from the jugular vein from 44 healthy cats owned by students and staff of the Veterinary Medical Teaching Hospital at Texas A&M University (VMTH-TAMU). This procedure was approved by the Clinical Research Review Committee (CRRC) at TAMU (#2005-27).

Information about breed or age of these cats was not available. Serum was separated and stored at -80ºC until required. Fecal samples were collected from 20 cats (age range: 1-16 years, median: 5.5 years; sex and sexual status: female spayed: 14, male neutered: 6; breeds: Domestic Short Hair: 11, Domestic Long Hair: 5, Domestic Medium Hair: 3, Norwegian Forest cat: 1) owned by staff and students of the VMTH-TAMU. This procedure was approved by the CRRC at TAMU (CRRC #2003-01). One gram of naturally-passed fecal material was collected from each cat's litter box once a day for three consecutive days, and placed in pre-weighed plastic tubes (Sarstedt, Nümbrecht, Germany). All samples were frozen immediately and stored at -20ºC by the cat's owner, and then stored at -80 ºC at the Gastrointestinal Laboratory.

Extraction of fecal fα_1-PI. The following protocol was used for the extraction of fα_1-PI from feces and was modified from a procedure originally described by Melgarejo et al. (1998). Each fecal sample was diluted 1:5 with phosphate-buffered saline (PBS), (Pierce, Rockford, Illinois), containing 5% newborn calf serum (NBCS), (Sigma-Aldrich, Inc., St. Louis, Missouri), and 0.01% thimerosal (Sigma-Aldrich, Inc., St. Louis, Missouri), pH 7.3. Each diluted fecal sample was homogenized using a mini vortexer (VWR, Suwanee, Georgia) for 20 minutes at room temperature. The homogenized sample was then centrifuged (Beckman centrifuge GS-R6, GMI, Ramsey, Minnesota) at 3,800 *g* for 20 minutes at 4ºC. The supernatant was removed using a tube filter (Fisher Scientific, Pittsburgh, Pennsylvania) and an aliquot of approximately 1.5 ml was placed into a microcentrifuge tube (Fisher Scientific, Pittsburgh, Pennsylvania). The tube was then centrifuged at 10,000 *g* for 30 minutes (Eppendorf centrifuge 5417C,

Eppendorf, Hamburg, Germany) and the final supernatant was transferred into another microcentrifuge tube (Fisher Scientific, Pittsburgh, Pennsylvania) and stored at -20ºC until analysis. For the ELISA, the processed fecal samples (1:5 dilution) were further diluted 1:400 with PBS, containing 5% NBCS and 0.01% thimerosal, pH 7.3, in order to reach a final dilution of 1:2,000.

Preparation of standards. A 1 mg/ml solution of purified fα_1-PI was diluted to concentrations of 4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 µg/L, using PBS containing 5% NBCS and 0.01% thimerosal, pH 7.3.

Enzyme-linked immunosorbent assay (ELISA). Each well of the microtiter plate (Thermo Electron Oy, Vantaa, Finland) was coated with 100 ng of purified capture antibodies diluted in 100 µl of 20 mM sodium carbonate bicarbonate buffer (Pierce, Rockford, Illinois), pH 9.6. The plate was incubated for one hour at 37ºC and then washed three times with PBS, containing 0.5% polyoxyethylenesorbitanmonolaurate (TWEEN-20, Sigma-Aldrich, Inc., St. Louis, Missouri). Next, 200 µl of SuperBlock® (Pierce, Rockford, Illinois) were added to each well in order to block nonspecific antibody binding sites. After incubation for one hour at 37ºC, the plate was again washed three times with PBS/TWEEN-20. One hundred µl of each of the seven standard solutions and 100 µl of the diluted fecal extracts or the diluted serum sample were added to the wells in duplicate and incubated for one hour at 37°C. For blanks the duplicate wells were loaded with 100 µl PBS, containing 5% NBCS and 0.01% thimerosal, pH 7.3. After three washes with PBS/TWEEN-20, 50 ng of biotinylated antibodies in 100 µL PBS containing 5% NBCS and 0.01% thimerosal, pH 7.3, were added to each well

and incubated for one hour at 37°C. The plate was again washed three times with PBS/TWEEN-20 and 5 ng of a streptavidin horseradish peroxidase (S-HRP) preparation (Sigma-Aldrich, Inc., St. Louis, Missouri) in 100 µL PBS containing 5% NBCS and 0.01% thimerosal, pH 7.3 was added to each well. After 30 minutes of incubation at 37ºC, the plate was once again washed three times with PBS/TWEEN 20 and a peroxidase substrate (TMB), (Pierce, Rockford, Illinois), was used to develop the plate. After eight minutes of incubation at room temperature, the reaction was stopped by adding 100 µl/well of a 4 M acetic acid, 0.5 M sulfuric acid solution. The microtiter plate was then read at 450 nm using an automated microplate reader and concentrations for fecal α_1-PI were calculated using the manufacturer's software (Labsystems Multiskan Ascent, Scientific Resources Southwest, Stafford, Texas). The standard curve was transformed in a log10/linear fashion and was based on a four parameter logistic fit using the formula $y=b+(a-b)/(1+xc)^d$.

Assay validation. The assay was validated by determination of the lowest detectable concentration, dilutional parallelism (linearity), spiking recovery of added α_1-PI (accuracy), and intra- and inter-assay coefficients of variation (reproducibility and precision, respectively). The validation procedures were performed separately for fecal extracts and serum samples. Statistical analyses were performed by commercially available software (GraphPad Prism 4, GraphPad Software Inc., San Diego, California).

The lower detection limit was determined by setting up ten duplicates of samples with zero fα_1-PI (suspended in PBS containing 5% NBCS[e] and 0.01% thimerosal, pH

7.3), and calculating the concentration of α_1-PI with an absorbance change equal to or greater than three standard deviations (SD) of these ten duplicates.

Linearity of the assay was determined by dilutional parallelism. Three serum samples from different cats were analyzed at dilutions of 1:500,000, 1:1,000,000, 1:2,000,000, 1:4,000,000, and 1:8,000,000. Three fecal samples from different cats were measured at dilutions of 1:1,000, 1:2,000, 1:4,000, and 1:8,000. The percentage of bias (in %) after each dilutional step was calculated from the observed to expected ratio. For this, the observed fα_1-PI concentration (value measured by the assay) was divided by the expected fα_1-PI concentration (value calculated according to the dilution factor), and multiplied by 100 (O/E x 100).

Accuracy of the assay was tested by spiking three serum samples and three fecal extracts each with known concentrations of purified fα_1-PI (2, 1, 0.5, 0.25, 0.125, 0.0625, and 0.03125 µg/L). Standard recovery (in %) after each spiking step was calculated from the obtained fα_1-PI concentration (value measured by the assay), divided by the expected fα_1-PI concentration (value calculated based on the added concentration of α_1-PI) and multiplied by 100 (O/E x 100).

Precision of the assay was determined by evaluation of three serum samples and three fecal extracts ten times within the same assay on the same day (intra-assay variability).

Reproducibility was determined by analyzing the same three serum samples and fecal extracts in ten consecutive assay runs (inter-assay variability). Reproducibility and precision were calculated as coefficient of variation (CV%= SD/ mean x 100%).

The reference interval for $f\alpha_1$-PI in serum was generated by calculating the central 95th percentile of 44 serum samples from healthy cats. The reference interval for $f\alpha_1$-PI in feces was established by evaluating the lower 97.5th percentile of the mean concentration of $f\alpha_1$-PI from three fecal samples collected on consecutive days from each of 20 healthy cats. Additionally, the mean coefficient of variation for the three fecal samples was calculated for this group of cats. Statistical significance was defined as $P< 0.05$.

RESULTS

Standard curves for the $f\alpha_1$-PI ELISA (Figure 1) were reproducible.

Lower detection limit. The lower detection limit of the assay was 0.02 g/L in serum and 0.04 µg/g in fecal samples.

Linearity (dilutional parallelism). Observed to expected (O/E) ratios for five serial dilutions of three serum samples ranged from 100.0 to 129.7% (mean ±SD: 112.2 ±9.9%) (Table 1). O/E ratios for four serial dilutions of fecal extracts ranged from 103.5% to 141.6% (mean ±SD: 115.6 ±12.8%) (Table 2).

Accuracy (spiking recovery). O/E ratios for serum and fecal samples spiked with different concentrations of purified $f\alpha_1$-PI ranged from 82.3 to 107.8% (94.7 ±7.6%) for serum samples (Table 3), and 78.5% to 148.7% (96.8 ±18.2%) for fecal samples (Table 4).

Precision (intra-assay variability). Coefficients of variation for intra-assay variability were 4.9, 6.4, and 7.9% for serum samples (Table 5), and 5.3%, 11.8%, and 14.2% for fecal samples (Table 6).

Reproducibility (inter-assay variability). The coefficients of variation for inter-assay variability were 6.8%, 10.0%, and 12.1% for serum samples (Table 5), and 7.7%, 10.2%, and 20.4% for fecal samples (Table 6).

Reference intervals. The reference interval for serum fα_1-PI as determined by calculation of the central 95th percentile was 0.64 to 1.4 g/L. Fecal fα_1-PI concentrations in all 60 samples from 20 healthy cats ranged from 0.04-1.9 µg/g, with a median of 0.42 µg/g (Figure 2). The reference interval for fecal fα_1-PI, as calculated by the lower 97.5th percentile for the average fα_1-PI concentrations of three fecal samples for each cat, was 0.04 to 1.6 µg/g of fecal material.

Three-day-variation of fα_1-PI concentrations for fecal extracts. The mean coefficient of variation for the three fecal samples was 46% (Figure 3). Lower mean α_1-PI concentrations had significantly higher coefficients of variation compared to higher mean α_1-PI concentrations ($P < 0.05$; r^2=0.206).

Table 1. Results for dilutional parallelism of serum samples for the feline α_1-PI ELISA. All three serum samples (I-III) were evaluated at the dilutions of 1:500,000; 1:1,000,000; 1:2,000,000; 1:4,000,000; and 1:8,000,000 and the observed (O) to expected (E) ratios were calculated.

	Dilution	**O g/L**	**E g/L**	**O/E %**
Serum I	500,000	2.96		
	1,000,000	1.61	1.48	108.78
	2,000,000	0.80	0.74	108.11
	4,000,000	0.43	0.37	116.22
	8,000,000	0.23	0.19	121.05
Serum II	500,000	1.09		
	1,000,000	0.56	0.55	101.82
	2,000,000	0.28	0.27	103.70
	4,000,000	0.14	0.14	100.00
	8,000,000	0.07	0.07	100.00
Serum III	500,000	2.02		
	1,000,000	1.31	1.01	129.70
	2,000,000	0.60	0.51	117.65
	4,000,000	0.31	0.25	124.00
	8,000,000	0.15	0.13	115.38

Table 2. Results for dilutional parallelism of fecal extracts for the feline α_1-PI ELISA. All three fecal samples (I-III) were evaluated at the dilutions 1:1,000; 1:2,000; 1:4,000; and 1:8,000 and the observed (O) to expected (E) ratios were calculated.

	Dilution	**O (µg/g)**	**E (µg/g)**	**O/E (%)**
Feces I	1000	0.830		
	2000	0.460	0.415	110.84
	4000	0.230	0.208	110.58
	8000	0.110	0.104	105.77
Feces II	1000	3.500		
	2000	2.010	1.750	114.86
	4000	1.160	0.875	132.57
	8000	0.620	0.438	141.55
Feces III	1000	0.290		
	2000	0.150	0.145	103.45
	4000	0.080	0.073	109.59
	8000	0.040	0.036	111.11

Table 3. Results for spiking recovery of serum samples for the feline α_1-PI ELISA. Seven different concentrations of feline α_1-PI (2, 1, 0.5, 0.25, 0.125, 0.0625, and 0.03125 g/L) were added to three different serum samples (I-III) and the observed (O) to expected (E) ratios were calculated.

	Concentration added in g/L	**O g/L**	**E g/L**	**O/E %**
Serum I	0.0000	1.37		
	0.0315	1.50	1.40	107.14
	0.0625	1.39	1.43	97.20
	0.1250	1.40	1.50	93.33
	0.2500	1.47	1.62	90.74
	0.5000	1.74	1.87	93.05
	1.0000	2.46	2.37	103.80
	2.0000	2.92	3.37	86.65
Serum II	0.0000	0.60		
	0.0315	0.60	0.63	95.24
	0.0625	0.59	0.66	89.39
	0.1250	0.66	0.73	90.41
	0.2500	0.70	0.85	82.35
	0.5000	0.94	1.10	85.45
	1.0000	1.69	1.60	105.63
	2.0000	2.14	2.60	82.31
Serum III	0.0000	1.12		
	0.0315	1.24	1.15	107.83
	0.0625	1.21	1.18	102.54
	0.1250	1.22	1.25	97.60
	0.2500	1.33	1.37	97.08
	0.5000	1.53	1.62	94.44
	1.0000	1.96	2.12	92.45
	2.0000	2.93	3.12	93.91

Table 4. Results for spiking recovery of fecal extracts of the feline α_1-PI ELISA. Seven different concentrations of feline α_1-PI (2, 1, 0.5, 0.25, 0.125, 0.0625, and 0.03125 µg/g) were added to three different fecal samples (I-III) and the observed (O) to expected (E) ratios were calculated.

	Concentration added in µg/g	**O (µg/g)**	**E (µg/g)**	**O/E (%)**
Feces I	0.0000	0.44		
	0.0315	0.56	0.47	119.15
	0.0625	0.57	0.50	114.00
	0.1250	0.56	0.56	99.12
	0.2500	0.75	0.69	108.70
	0.5000	0.83	0.94	88.30
	1.0000	1.20	1.44	83.33
	2.0000	2.01	2.44	82.38
Feces II	0.0000	2.11		
	0.0315	2.15	2.14	100.47
	0.0625	1.92	2.17	88.48
	0.1250	1.91	2.24	85.27
	0.2500	1.97	2.36	83.47
	0.5000	2.10	2.61	80.46
	1.0000	2.44	3.11	78.46
	2.0000	3.41	4.11	82.97
Feces III	0.0000	0.12		
	0.0315	0.14	0.15	93.33
	0.0625	0.19	0.18	105.56
	0.1250	0.32	0.25	128.00
	0.2500	0.55	0.37	148.65
	0.5000	0.53	0.62	85.48
	1.0000	0.97	1.12	86.61
	2.0000	1.90	2.12	89.62

Table 5. Precision and reproducibility of serum samples for the feline α_1-PI ELISA. This table shows coefficients of variation (CV) for three different serum samples (S I-III) for the intra-assay and inter-assay variability of the feline α_1-PI ELISA. Serum samples were evaluated ten times within the same assay run (intra-assay) and ten times in consecutive runs (inter-assay).

	Number of repeats	**Mean (g/L)**	**Standard deviation (g/L)**	**Coefficient of variation (%)**
Intra-assay variability				
S I	10	0.56	0.03	4.9
S II	10	1.14	0.07	6.4
S III	10	1.45	0.11	7.9
Inter-assay variability				
S I	10	0.54	0.04	6.8
S II	10	1.07	0.11	10.0
S III	10	1.44	0.17	12.1

Table 6. Precision and reproducibility of fecal extracts for the feline α_1-PI ELISA. This table shows coefficients of variation (CV) for three different fecal extracts (F I-III) for the intra-assay and inter-assay variability of the feline α_1-PI ELISA. Fecal extracts were evaluated ten times within the same assay run (intra-assay) and ten times in consecutive runs (inter-assay).

	Number of repeats	**Mean (µg/g)**	**Standard deviation (µg/g)**	**Coefficient of variation (%)**
Intra-assay variability				
F I	10	0.110	0.016	14.2
F II	10	0.387	0.045	11.8
F III	10	1.997	0.107	5.3
Inter-assay variability				
F I	10	0.119	0.024	20.4
F II	10	0.442	0.034	7.7
F III	10	1.925	0.196	10.2

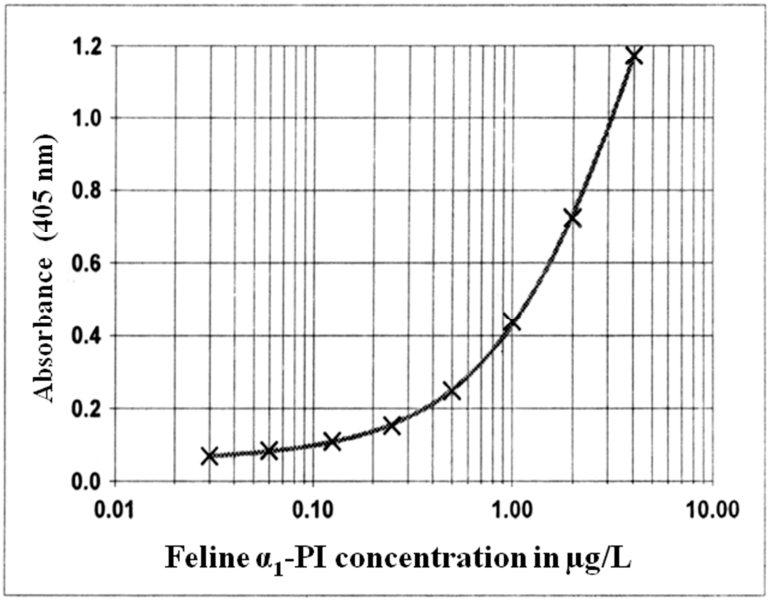

Figure 1. Representative standard curve for the feline α_1–PI ELISA. The curve is based on a four parameter logistic fit curve [y=b+(a-b)/(1+xc)d] transformed in a log10/linear fashion. The seven standards had the following concentrations: 4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 µg/L.

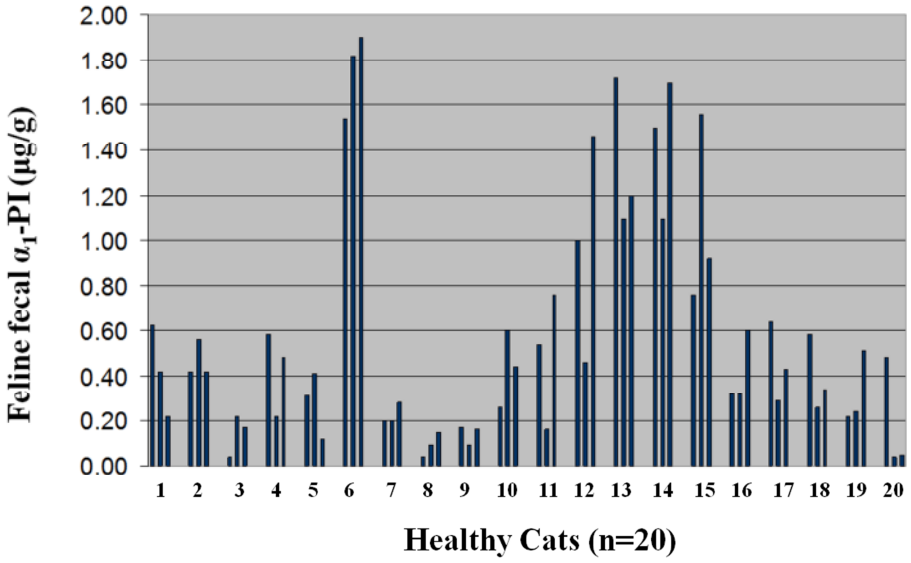

Figure 2. Variation of fecal α_1–PI concentrations in three fecal samples of 20 healthy cats. This figure shows the day to day variation of fecal α_1-PI concentrations of the three fecal samples collected from each of 20 healthy cats on consecutive days. Cats no. 3, 8, and 20 had one fecal sample each with a concentration in the undetectable range, designated here as a concentration equal to the lower range of detection of 0.04 µg/g. The coefficient of variation for the three fecal samples was significantly higher in samples with lower mean α_1-PI concentrations ($P< 0.05$; $r^2=0.206$), while the mean coefficient of variation for all 20 cats was 46%.

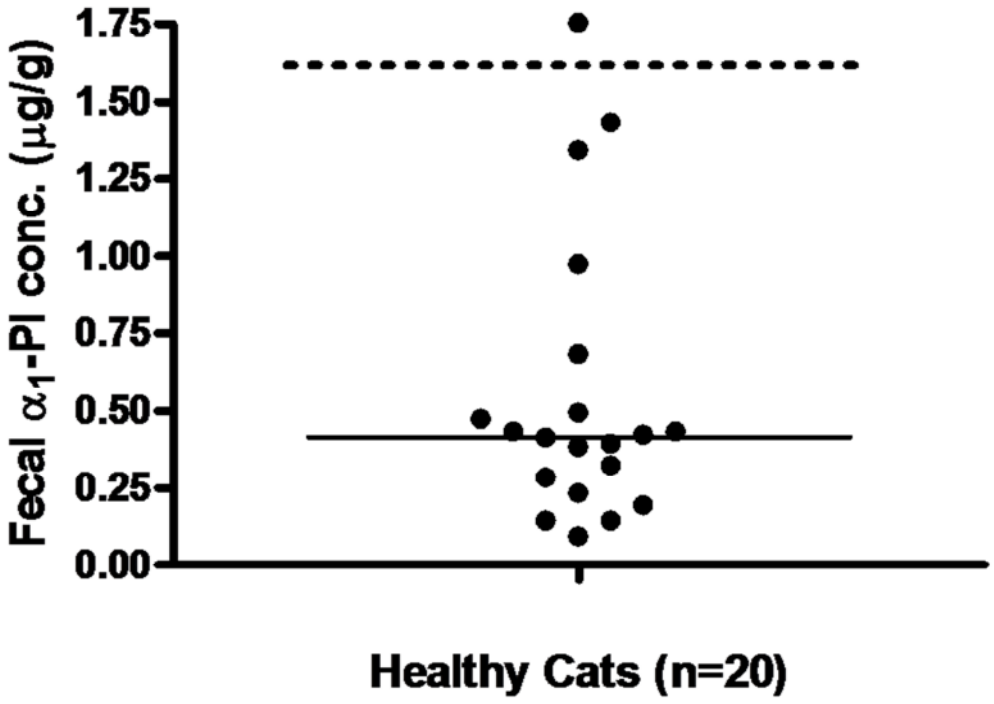

Figure 3. Mean fecal α_1–PI concentrations in healthy cats. This figure illustrates the mean fecal α_1-PI concentrations of the 3 fecal samples collected each from 20 healthy cats. The median of the mean fecal α_1-PI concentrations of the 20 healthy control cats was 0.42 µg/g of fecal material as shown as the straight line in the figure. The dotted line in this figure shows the upper limit of reference interval (1.6 µg/g).

DISCUSSION

An ELISA for the quantification of fα_1-PI in serum and feces from cats was successfully developed. In contrast to the previously developed radioimmunoassay, the ELISA also has sufficient analytical performance to measure fα_1-PI in fecal samples, and, to the authors' knowledge, represents the first assay available for this purpose.

Alpha$_1$-PI is the prototype of the serine proteinase inhibitors, a family of proteins known to be very abundant in mammalian plasma (Hibbetts et al, 1999). Although determined with different analytical methods, the reference interval for serum α_1-PI established for cats in our study (0.64-1.4 g/L) was comparable to reference intervals of other species, e.g., humans (1.1-2.1 g/L) (Ward et al. 1985), dogs (0.9-2.11 g/L) (Melgarejo et al. 1998), or horses (1.3-1.6 g/L) (Patterson et al., 1991). Because of the relative abundance of α_1-PI in blood, it was necessary to greatly dilute serum samples from healthy cats (e.g., 1:1,000,000), in order to be able to measure its concentration within the range of standards for our assay (0.0625 to 4 µg/L). In contrast, the concentration of α_1-PI in feces is only a fraction of that in serum in healthy cats, and therefore, dilutions of fecal samples for our assay were much lower compared to serum samples (e.g., 1:2000).

The lower detection limit of the assay was determined to be 0.02 g/L for serum samples, which appears to be an acceptable value considering the established reference interval for serum samples of 0.64-1.4 g/L. This newly established reference interval for serum samples from 44 healthy cats is slightly higher, but comparable to the previously established reference interval for serum α_1-PI concentrations of 0.25 to 0.6 g/L based on

a radioimmunoassay using a different group of 50 healthy cats (Fetz et al., 2004). Differences between these reference intervals are readily explained by the different methodologies for the two immunoassays.

Given that fecal samples were extracted from grams of fecal matter, the unit µg/g was chosen for expressing the concentration of α_1-PI in fecal extracts. Compared to serum samples and considering the different unit, the lower detection limit of the assay for fecal extracts was similarly low at 0.04 µg/g. As expected, the concentration of α_1-PI in fecal extracts was occasionally below the value of the lower limit of detection in a few of the healthy cats, and these values were therefore defined as <0.04 µg/g.

The measurement of fecal α_1-PI concentrations in healthy cats resulted in the establishment of a relatively narrow control range (<0.04-1.6 µg/g). Fecal α_1-PI concentrations in healthy cats were lower compared to the control range of fecal α_1-PI concentrations previously established in dogs (0.23 to 5.67 µg/g). While the canine assay is also an ELISA, discrepancies in reference intervals may be explained by differences of assay properties. Alternatively, this may suggest differences in the physiological distribution of α_1-PI in these two species. Heilmann et al. (2011) recently developed and analytically validated a radioimmunoassay (RIA) for fecal canine α_1-PI. This assay agreed well with the previously established ELISA for this species.

Dilutional parallelism studies are useful to demonstrate the relative recovery after dilution and thus the linearity of the assay. Observed to expected ratios for dilutional parallelism for serum and fecal samples had a mean ±SD of 112.2% ± 9.9 and 115.6% ± 12.8, respectively. While 100% is the ideal value, our results revealed acceptable, slight

over-recovery on dilution of both serum and fecal samples. The reason for the over-recovery in our assay is not clear, but this may be generally explained by the presence of high-affinity antibodies (Crowther, 2001).

Accuracy, representing the ability of an assay to measure the true value of an analyte, is tested by spiking recovery. Both negative and positive biases were noted over the range of serum and fecal samples, likely indicating the presence of both proportional and constant biases. Furthermore, for both sample types, there appears to be a tendency for a negative bias when higher α_1-PI concentrations are added. While the degree of bias is more pronounced with fecal extracts compared to serum samples, the overall mean bias and SD of 94.7 ±7.6 for serum samples and 96.8 ±18.2 for fecal samples appear acceptable.

Precision and reproducibility may be considered as the most important technical aspects of an immunoassay (Deshpande, 1996). Intra- and inter-assay coefficients of variation (CV) of equal to or less than 15% indicate adequate precision and reproducibility for acceptable assay performance for clinical use of an assay (Crowther, 2001). An assay's CV will typically vary with the analyte's concentration, with higher CV values often found at the lower and upper limits of the analytical range of the assay (Stockham and Scott, 2008). The validation of our assay revealed greater precision and reproducibility for lower fα_1-PI concentrations in serum. In contrast, fecal extracts showed greater precision and reproducibility at higher fα_1-PI concentrations. Precision especially decreased for the lowest fecal fα_1-PI concentrations for both intra-and interassay runs (CV% 14.2 and 20.4, respectively). While potential concern from these

values for appropriate assay precision in low concentration samples cannot be denied, impact on the clinical utility of the assay may not be as great because protein-losing enteropathies are expected to cause elevated concentrations of this protein in feces, where assay precision is acceptable.

We further showed that fα_1-PI concentrations in feces can vary significantly in healthy cats in consecutive samples of the same individual (mean coefficient of variation: 46%). The large coefficients of variation suggests that more than one fecal sample should be collected in order to increase the accuracy in future clinical studies regarding gastrointestinal loss of this protein.

An important limit of this study is the low number of the reference sample group for both fecal and serum samples. For our study, 44 serum and 60 fecal samples (the latter consisting of three samples from 20 individual cats each) were available at the time of the assay validation. Based on statistical recommendations, reference intervals should be established from at least 120 independent observations; however, if a Gaussian distribution is present, as was the case for our samples, a minimum of 60 samples from qualified individuals is generally regarded as sufficient (Lumsden, 2000). As reduced number of individuals may lead to lower statistical power to detect significant differences between healthy and diseased animals, the establishment of a new reference interval based on serum and fecal samples from at least 60 individuals is needed when conducting the clinical evaluation of this assay. Furthermore, we did not perform studies on the effect of storage or repeated freeze-thaw cycles on recovery of fα_1-PI. Preliminary studies have shown that purified fα_1-PI appears to be stable for >3 years when stored at -

80ºC. However, studies regarding stability of this protein in serum, feces or fecal extracts at more commonly used storage temperatures are still warranted.

CHAPTER III

EVALUATION OF FECAL ALPHA$_1$-PROTEINASE INHIBITOR CONCENTRATIONS IN CATS WITH IDIOPATHIC INFLAMMATORY BOWEL DISEASE AND CATS WITH GASTROINTESTINAL NEOPLASIA

OVERVIEW

Idiopathic inflammatory bowel disease (IBD) and gastrointestinal (GI) lymphoma are common disorders in cats. The aim of this study was to evaluate fecal α_1-PI concentrations, a marker of GI protein loss, in cats with histopathological evidence of GI inflammation or GI neoplasia.

Twenty cats with clinical signs of chronic GI disease and 20 healthy control cats were enrolled. Diagnostic endoscopies with histopathological evaluation were performed on the 20 diseased cats. Two groups of cats were assembled [Group A (n=8): mild to moderate IBD, Group B (n=12): severe IBD or neoplastic disease]. Fecal α_1-PI concentrations and serum concentrations of total protein, albumin, globulin, cobalamin, folate, pancreatic lipase immunoreactivity, and trypsin-like immunoreactivity were determined.

Nineteen of the 20 diseased cats had elevated fecal α_1-PI concentrations, ranging from 1.9 to 233.6 µg/g (normal range: ≤1.6 µg/g). Fecal α_1-PI concentrations were statistically significantly different between healthy cats and cats of Group A (median: 3.9 µg/g, range: 1.3 to 9.2 µg/g, P<0.001) or cats of Group B (median: 20.6 µg/g, 4.3 to

233.6 µg/g; $P<0.001$), and between cats of Groups A and B ($P<0.01$). Hypoalbuminemia, hypoproteinemia, and hypocobalaminemia were detected in 88%, 83%, and 56% of the diseased cats, respectively.

This study suggests that increased fecal α_1-PI concentrations in association with low serum albumin and total protein concentrations may be a common finding in cats with IBD or GI neoplasia. Furthermore, α_1-PI concentrations appear to be higher in cats with severe IBD or confirmed GI neoplasia when compared to cats with mild to moderate IBD.

INTRODUCTION

Idiopathic inflammatory bowel disease (IBD) and gastrointestinal (GI) lymphoma are commonly diagnosed disorders in cats with chronic clinical signs of GI disease. Idiopathic inflammatory bowel disease refers to a group of poorly understood chronic gastroenteropathies that are characterized by infiltration of the GI mucosa with inflammatory cells. Infiltrating inflammatory cells most commonly consist of lymphocytes and/or plasma cells, while additional infiltrates of neutrophils, histiocytes, and eosinophils also may be observed (Wilcock, 1992; Waly et al., 2004) The etiopathogenesis of IBD in cats remains largely unclear, but it is thought to be related to complex interactions between host susceptibility, mucosal immunity, and the enteric microbiota (Jergens et al., 1992).

Gastrointestinal lymphoma is considered one of the most commonly diagnosed neoplasms in cats and its prevalence has increased significantly in the past two decades

(Wilson, 2008). Clinical signs of both IBD and GI lymphoma are non-specific, and commonly include vomiting, diarrhea, weight loss, anorexia, or any combination of these signs. The diagnosis of IBD or GI lymphoma is generally made by excluding other GI disorders with similar clinical signs, such as dietary hypersensitivity, hyperthyroidism, parasitism, or hepatic, pancreatic, or renal disease, and histopathologic evaluation of intestinal biopsy samples.

In dogs, IBD, GI lymphoma, or any other chronic gastroenteropathy producing inflammation, vascular congestion, or bleeding may be associated with transmucosal loss of plasma proteins, protein malabsorption, or both (Murphy et al., 2003). In protein-losing gastroenteropathies (PLE), GI disease results in nonselective protein loss. Clinicians may only recognize severe cases of PLE, when GI protein loss has exceeded albumin production by the liver, thus leading to hypoalbuminemia or a decreased serum total protein concentration. In the veterinary literature there has been little mention of protein loss associated with gastroenteropathies in cats. This may reflect the difficulties in diagnosing GI protein loss in this species due to the lack of suitable diagnostic methods, or the failure to recognize the presence of this condition in cats until other, more severe manifestations of their GI disease are observed.

Gastrointestinal protein loss in humans and dogs can be diagnosed by the measurement of the endogenous protein α_1-proteinase inhibitor (α_1-PI) in feces (Thomas et al., 1981; Karbach et al., 1983; Murphy et al., 2003). In healthy individuals, only minute amounts of α_1-PI are present in the gastrointestinal lumen. Like other plasma proteins, α_1-PI may be lost into the GI lumen as a result of gastrointestinal disease.

Unlike most other plasma proteins, including albumin, α_1-PI is a proteinase inhibitor and thus able to resist degradation by digestive or bacterial proteolytic enzymes and therefore can be detected in feces by use of an immunoassay (Suchodolski and Steiner, 2003).

We have recently reported on the development and analytical validation of an ELISA for the measurement of α_1-PI concentrations in fecal samples of cats (Burke et al., 2012). The aim of this study was to evaluate fecal α_1-PI concentrations in cats with a histopathological diagnosis of GI inflammation or cats with GI neoplasia. We hypothesize that fecal α_1-PI concentrations would be higher in cats with chronic GI disease compared to healthy cats.

MATERIALS AND METHODS

Cats and sample collection. For the control group, 20 healthy cats owned by staff and students of the College of Veterinary Medicine and Biomedical Sciences at Texas A&M University were enrolled. From each cat one gram of naturally-passed fecal material was collected out of the litter box at home and placed in a pre-weighed plastic tube. All samples were frozen immediately at -20ºC by the cat's owner and then stored at -80 ºC at the Gastrointestinal Laboratory. Sera were not available from these cats.

For the diseased group, a total of 20 cats with signs of chronic GI disease (>6 weeks duration) were entered into the study. All cats were owned by clients of the Animal Medical Center (AMC) in New York City and recruited for an unrelated study. All cats exhibited various combinations of clinical signs, such as vomiting, diarrhea,

weight loss, and anorexia at the time of initial presentation (Table 1). Diagnostic tests evaluated in these cats included complete blood count, serum biochemistry profile, urinalysis, fecal direct smear and flotation, serum total T4, and FeLV/ FIV testing. None of the cats was on any treatments for at least three weeks before enrollment. After enrollment approximately one to two grams of naturally-passed fecal material and one blood sample were collected from each cat. Serum was obtained from the blood samples. Both fecal and serum samples were frozen immediately and kept at -80 ºC until they were shipped frozen to the Gastrointestinal Laboratory at Texas A&M University.

Biopsy procedure and histopathological examination. All cats of the diseased group underwent gastroduodenoscopy in order to obtain gastric and intestinal biopsies. Three cats also underwent colonoscopy in order to obtain colonic biopsies. From two cats, liver biopsies were obtained percutaneously under ultrasound-guidance using a disposable 18 gauge tru-cut biopsy needle (Bard Biopsy Systems, Tempe, AZ).

Cats were premedicated with glycopyrrolate (0.01 mg/kg, IM) or atropine (0.02 mg/kg, IM). Anesthesia was induced with propofol (3 to 6 mg/kg, IV) and diazepam (0.5 mg/kg, IV), and maintained with inhaled isoflurane or sevoflurane and oxygen. Gastroduodenoscopy and colonoscopy were performed by a single endoscopist. A video gastroscope (GIF 100, Olympus America, Mellville, NY) with a 100-cm insertion tube, 9.5-mm outer diameter, and a 2.8-mm working channel as well as a 2.6-mm flexible biopsy forceps were used. Mucosal surfaces were evaluated and gross lesions were recorded. Multiple representative specimens were obtained for histological evaluation in each cat.

Biopsy specimens were labeled and placed in jars containing 10% formalin solution and submitted for histopathological evaluation. The histopathological evaluation was performed by one of four pathologists. Specimens were evaluated for inflammatory, neoplastic, or other lesions. Idiopathic inflammatory bowel disease was diagnosed based on varying degrees of infiltration by inflammatory cells (mainly mixed populations of lymphocytes and plasma cells, and small, mature lymphocytes being most prominent) within the mucosa and submucosa without any identifiable underlying cause for the inflammatory infiltrate. Histopathological grading was performed as described by Hart et al. (1994). Mild grade consists of mild to moderate diffuse infiltration of the mucosa with inflammatory cells with no architectural changes. Moderate grade consisted of moderate diffuse infiltration of the mucosa with inflammatory cells, mild to moderate edema or fibrosis of the mucosa, slight villus blunting with irregular crypts, and occasional dilated lacteals. Severe grade consisted of severe diffuse infiltration of the mucosa with inflammatory cells, variable epithelial flattening and erosion, moderate to severe edema and fibrosis, fusion and blunting of villi and dilated lacteals, crypt hyperplasia, and crypt abscessation. Gastrointestinal lymphoma was diagnosed based on marked mucosal infiltration and expansion by a mainly monomorphic population of lymphoid cells with much fewer other inflammatory cells present. Based on the results of the histopathologic examination, cats were classified into the following two groups: Group A included cats with mild or moderate enteritis (histologic grade 1, HG 1) and Group B were cats with a HG >1, which included the following diagnoses: severe

enteritis (HG 2), an inconclusive diagnosis of either severe enteritis or GI lymphoma (HG 2-3), or definite diagnosis of GI lymphoma or other GI neoplasia (HG 3).

Fecal and serum assays. Fecal α_1-PI concentrations were measured in the feces from each cat with a previously developed and validated sandwich-ELISA (Burke et al., 2011). A reference interval for feline α_1-PI in feces was calculated by evaluating the lower 97.5^{th} percentile of feline α_1-PI concentration from fecal samples collected from the 20 healthy cats. Serum samples were available from 18 of the 20 diseased cats (11/12 of Group A, and 7/8 of Group B). Serum total protein, albumin, and globulin concentrations were measured with a Hitachi 911 (Diamonds Diagnostics, Holliston, MA, USA), at the Texas Veterinary Medical Diagnostic Laboratory, College Station, Texas. Serum concentrations for pancreatic lipase immunoreactivity (PLI), and trypsin-like immunoreactivity (TLI) were measured by in-house-ELISA and in-house RIA, respectively. Serum cobalamin and folate were measured using an Immulite 2000 (Siemens Healthcare Diagnostic Inc., Tarrytown, NY, USA). Other clinicopathological findings were not available.

Statistical evaluation. Commercially available software (GraphPad Prism 4, Graph Pad, San Diego, California) was used for all statistical analyses. Data were tested for normal distribution with the D'Agostino and Pearson omnibus test. Fecal α_1-PI concentrations were transformed to log10 values for normal distribution and compared between healthy cats, cats of Group A, and cats of Group B with a 1-way ANOVA test, followed by Tukey's multiple comparison tests. Serum concentrations for total protein, albumin, globulin, cobalamin, folate, PLI, and TLI were compared between cats of

Group A and cats of Group B with a Mann-Whitney test. For all statistical analyses, statistical significance was defined as $P< 0.05$.

RESULTS

Signalment of healthy cats. Age range: 1-16 years (median: 5.5 years); sex and sexual status: female spayed: 14, male neutered: 6; breeds: Domestic Short Hair: 11, Domestic Long Hair: 5, Domestic Medium Hair: 3, Norwegian Forest cat: 1.

Signalment of diseased cats. Ages of cats in Group A ranged from 4 to 17 years (median: 9 years), all cats were Domestic Shorthair cats (DSH), 5 cats were male neutered and 3 cats were female spayed (Table 7). Ages of cats in Group B ranged from 4 to 16 years (median: 10 years), 5 were male neutered and 7 female spayed, 10 DSH, 1 American Shorthair, 1 Maine Coon (Table 7).

Clinical signs. The most common presenting clinical sign in both groups was weight loss (18/20; 90%), followed by diarrhea (15/20; 75%), vomiting (14/20; 70%), and anorexia (4/20; 20%). Most cats had multiple clinical signs and the most commonly reported combination of clinical signs for both groups was vomiting, diarrhea, and weight loss (7/20; 35%) (Table 7). The frequency of clinical signs for cats in Group A was: weight loss (7/8, 88%), vomiting (6/8, 75%), diarrhea (5/8, 63%), anorexia (1/8, 13%). The frequency of clinical signs for cats in Group B was: weight loss (11/12, 92%), diarrhea (10/12, 83%), vomiting (9/12, 75%), anorexia (3/12, 25%).

Histopathological and clinical diagnoses. Histopathological grading, lesion localization, and histopathological diagnoses of the cats with chronic GI disease are

shown in Table 8. Of the 20 diseased cats, a total of 8 cats were diagnosed with mild or moderate IBD and included in Group A (mild, n=3; mild to moderate, n=4; moderate, n=1) based on results of the histopathological examination. The remaining 12 cats were included in Group B and had the following diagnoses: severe IBD (n=2), inconclusive diagnosis of either severe IBD or GI lymphoma (n=5), confirmed GI neoplasia (n=5, including GI lymphoma, n=3; colonic carcinoma, n=2).

Fecal α_1-PI concentrations. In the healthy cats, fecal α_1-PI concentrations ranged from 0.04 (or undetectable value) to 1.72 µg/g (median: 0.51 µg/g). The reference interval based on the lower 97.5th percentile was calculated to be ≤ 1.6 µg/g. Of the 20 diseased cats, 19 cats had increased fecal α_1-PI concentrations (median: 7.5 µg/g, range: 1.9 to 233.6), while one cat had fecal α_1-PI concentrations (1.3 µg/g) within the reference interval of the healthy control cats (≤ 1.6 µg/g) (Table 7). Both cats of Group A (median: 3.9 µg/g, range: 1.3 to 9.2 µg/g) and those of Group B (median: 20.6 µg/g, range: 4.3 to 233.6 µg/g) had statistically significant different fecal α_1-PI concentrations compared to healthy cats (Tukey's multiple comparison test; $P<0.001$ and $P<0.001$, respectively) (Figure 4). Also, fecal α_1-PI concentrations were statistically significant different between Group A and B (Tukey's multiple comparison test; $P<0.01$). When the two extreme values of cats in Group B (B-1: 233.6 µg/g, B-4: 179.2 µg/g) were excluded before statistical evaluation, statistical significance persisted (Healthy cats vs. Group A, $P<0.001$; Healthy cats vs. Group B, $P<0.001$; Group A vs Group B, $P<0.05$).

Serum total protein concentrations. Serum total protein concentrations for both diseased groups ranged from 4.1 to 6.8 g/dL (median: 5.6 g/dL, reference interval: 6.5-8.9 g/dL) (Table 7). Median serum total protein concentration was 6.2 g/dL (range: 4.4-6.8 g/dL) for Group A and 5.2 g/dL (range: 4.1-6.2 g/dL) for Group B. Serum total protein concentrations were statistically significantly different from each other (Mann-Whitney; *P*=0.0297). Hypoproteinemia was present in 15 of 18 cats (all: 83%; Group A: 4/7, 57%; Group B: 11/11, 100%).

Serum albumin concentrations. Serum albumin concentrations for both diseased groups ranged from 1.6-3.3 g/dL (median: 2.65 g/dL; reference interval: 3.2-4.7 g/dL) (Table 7). Median serum albumin concentrations were 2.6 g/dL (range: 2.4-3.3 g/dL) for Group A and 2.7 g/dL (range: 1.6-3.0 g/dL) for Group B. However, there was no statistical significance of serum albumin concentrations between cats of Groups A and B (*P*= 0.496). Hypoalbuminemia was present in 16 of 18 cats (88%; Group A: 5/7, 71%; Group B: 11/11, 100%)

Serum globulin concentrations. Serum globulin concentrations for both diseased groups ranged from 2.0-4.1 g/dL (median: 2.5 g/dL; reference interval: 2.8-4.8 g/dL) (Table 7). Median serum globulin concentration was 3.5 g/dL (range: 2.0-4.1 g/dL) for Group A and 2.5 g/dL (range: 2.1-3.6 g/dL) for Group B. Serum globulin concentrations were not statistically significantly different between both groups (Mann-Whitney; *P*=0.0632). Hypoglobulinemia was present in 7 of 18 cats (38.8%; Group A: 1/7, 14%; Group B: 6/11, 55%). Concurrent hypoalbuminemia was present in all cases

of hypoglobulinemia (panhypoproteinemia, 38.8%; Group A: 1/7, 14%; Group B: 6/11, 55%).

Serum cobalamin and folate concentrations. Hypocobalaminemia (<290 ng/L) was present in 10 of 18 cats (56%; Group A: 1/7, 14%; Group B: 9/11, 81%) (Table 9). In Group B, all cats with a diagnosis of severe IBD, severe IBD or GI lymphoma, or GI lymphoma were hypocobalaminemic, while the two cats with colonic carcinoma had cobalamin concentrations within the reference interval (290-1500 ng/L). Cobalamin concentrations of Group B cats (median: 139 ng/L, range: 99-1201 ng/L) were significantly lower than for cats of Group A (median: 994 ng/L, range: 153-1201 ng/L; Mann-Whitney; *P*=0.0098). Serum folate concentration was increased in two cats from Group A, decreased in one cat from Group B, and within the reference interval in the remaining cats. No statistically significant difference was observed for serum folate concentration between the two groups (Mann-Whitney, *P*=0.4688).

Serum trypsin-like immunoreactivity (TLI) and serum pancreatic lipase immunoreactivity (PLI)) concentrations. Serum TLI concentrations ranged from 29.1 to 206.7 µg/L (median: 84.7 µg/L; reference interval: 12-82 µg/L) in both groups (Group A: median: 72.7 µg/L, range: 47.9 - 145.9 µg/L; Group B: median: 102.3 µg/L, range: 29.1-206.7 µg/L) (Table 9). Serum TLI concentration was increased in 9 of 18 cats (50%) of all diseased cats. More specifically, TLI was increased in 2 of 7 cats (29%) of Group A, and 7 of 11 cats of Group B (64%). No statistically significant difference was reached for serum TLI concentrations between the two groups (Mann Whitney, *P*=0.5261).

Serum PLI concentrations ranged from <2 to 54.8 µg/L (median: 6.1 µg/L; reference interval: 2-6.8 µg/L) in both groups (Group A: median: 4.8 µg/L, range: <2 to 8.1 µg/L; Group B: median 6.1 µg/L, range: 3.7 to 54.8 µg/L,). Serum PLI concentration was increased in 7 of 18 cats (39%) of both groups, which included 2 cats (29%) of Group A, and 5 cats (45%) of Group B. The serum PLI concentration was strongly suggestive of pancreatitis (>12 µg/L) in 3 cats of Group B. No statistically significant difference was reached for serum PLI concentrations between the two groups (Mann Whitney, P=0.1451).

Concurrent increases in serum TLI and PLI was present in 6 of 11 cats from both groups [Group A: 1/7; Group B: 5/11]. Increased TLI concentration without concurrent increase of the PLI concentration was present in 3 cats, while a mildly increased PLI concentration without concurrent increased TLI concentration was noted in one cat.

Table 7. Signalment, clinical signs, and serum concentrations of albumin, globulin, total protein, and fecal α_1-PI concentrations of the cats with chronic GI disease.

Cat ID	Age (years)	Sex	Breed	Clinical signs	Total Protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	Fecal α_1-PI (µg/g feces)
					ref: 6.5-8.9 g/dL	**ref: 3.2-4.7 g/dL**	**ref: 2.8-4.8 g/dL**	**ref: ≤1.6 µg/g)**
GROUP A								
A-1	8	MN	DSH	VDW	6.5	3.3	3.2	***1.9***
A-2	7	MN	DSH	VDW	***6.1***	***2.6***	3.5	***9.2***
A-3	5	MN	DSH	VW	***4.4***	***2.4***	***2.0***	***3.6***
A-4	10	MN	DSH	V	***6.0***	***2.8***	3.2	***6.1***
A-5	4	FS	DSH	DW	***6.2***	***2.4***	3.8	1.3
A-6	12	FS	DSH	VWA	6.6	***2.5***	4.1	***2.6***
A-7	17	FS	DSH	VDW	6.8	3.2	3.6	***4.2***
A-8	11	MN	DSH	DW	NA	NA	NA	***7.2***
GROUP B								
B-1	9	FS	DSH	VDWA	***4.8***	***2.7***	***2.1***	***233.6***
B-2	10	MN	DSH	VDWA	***5.7***	***2.8***	3.1	***4.3***
B-3	15	MN	DSH	VWA	***6.2***	***3***	3.2	***7.7***
B-4	12	MN	DSH	W	***4.4***	***2.1***	***2.3***	***179.2***
B-5	9	FS	DSH	VD	***5.2***	***2.9***	***2.3***	***6.9***
B-6	11	MN	DSH	VDW	NA	NA	NA	***9.8***
B-7	16	FS	DSH	VDW	***4.1***	***1.9***	***2.2***	***5***
B-8	10	FS	ASH	VDW	***4.1***	***1.6***	***2.5***	***27.7***
B-9	8	FS	DSH	DW	***5.4***	***2.1***	3.3	***14.2***
B-10	4	FS	DSH	VDW	***4.7***	***2.6***	***2.1***	***30.8***
B-11	11	MN	MNC	VDW	***5.5***	***2.7***	2.8	***27***
B-12	9	FS	DSH	DW	***6.4***	***2.8***	3.6	***27.1***

Values outside of the reference interval are presented in bold and italic. MN, male neutered; FS, female spayed; DSH, Domestic Short Hair; ASH, American Short Hair; MNC, Maine Coon; V, vomiting; D, diarrhea; W, weight loss; A, anorexia; NA, not available

Table 8. Histopathological grading, lesion localization, and histopathological diagnoses of the cats with chronic GI disease.

Cat ID	HG*	Lesion localization**	Morphologic diagnosis of endoscopic biopsy specimens
GROUP A			
A-1	1	g1, d1	Minimal lymphocytic gastritis; **mild lymphoplasmacytic enteritis**
A-2	1	g1, d1	Mild chronic gastritis; **mild to moderate lymphoplasmacytic enteritis**
A-3	1	g1, d1	Mild lymphocytic gastritis with helical bacteria; **mild lymphoplasmacytic enteritis**
A-4	1	g1, d1	Minimal lymphocytic gastritis; **mild lymphoplasmacytic enteritis**
A-5	1	g1, c1	Mild to moderate chronic lymphocytic gastritis; **moderate lymphoplasmacytic colitis**, **severe chronic cholangiohepatitis/portal hepatitis**
A-6	1	g1, d1	Minimal lymphocytic gastritis with helical bacteria; **mild to moderate plasmacytic enteritis**
A-7	1	g1, d1	Mild chronic gastritis; **mild enteritis**
A-8	1	g1, d1	Mild gastritis; **moderate lymphoplasmacytic enteritis**, mild hepatic vacuolar degeneration
GROUP B			
B-1	2	g1, d2	Mild gastritis with helical bacteria; **severe lymphoplasmacytic enteritis**
B-2	2	g1, d2	Multifocal lymphocytic gastritis; **severe lymphocytic enteritis**
B-3	2 or 3	g1, d2-3	Moderate lymphocytic gastritis with surface helical bacteria; **severe diffuse lymphocytic enteritis or lymphoma**
B-4	2 or 3	g1, d2-3	Chronic moderate lymphocytic gastritis with helical bacteria; **severe diffuse lymphocytic enteritis or lymphoma**
B-5	2 or 3	g1, d2-3	Minimal lymphocytic gastritis; **severe diffuse enteritis/ suspect lymphoma**
B-6	2 or 3	g2-3, d2-3, c2-3	**Severe gastroenteritis or lymphoma**
B-7	2 or 3	g1, d2-3	Mild chronic gastritis; **severe gastroenteritis or lymphoma**
B-8	3	g3, d3	**Gastric and duodenal lymphoma**
B-9	3	d3	**Duodenal lymphoma**
B-10	3	g1, d3	Mild lymphocytic gastritis; **duodenal lymphoma**
B-11	3	g2, d1, c3	Severe chronic active ulcerative lymphoplasmacytic gastritis with helical bacteria, mild lymphoplasmacytic enteritis; **colonic adenocarcinoma**
B-12	3	g1, d1, c3	Mild gastritis; moderate lymphoplasmacytic proximal enteritis; severe diffuse lymphocytic distal enteritis; **colonic carcinoma**

Clinically most significant diagnoses are indicated in bold. *HG, histopathologic grade: 1, mild to moderate inflammation; 2, severe inflammation; 2 or 3, severe inflammation or neoplasm (inconclusive); 3, neoplasm. **g, gastrum; d, duodenum; c, colon; numbers correspond to the description of the histopathologic grade (see above).

Table 9. Serum concentrations of cobalamin, folate, trysin-like immunoreactivity (TLI), and pancreatic lipase immunoreactivity (PLI) for the cats with chronic GI disease.

Cat ID	Serum cobalamin (ng/L) ref: >290 ng/L	Serum folate (µg/L) ref: 9.7-21.6 µg/L	Serum TLI (µg/L) ref: 12-82 µg/L	Serum PLI (µg/L) ref: 2-6.8 µg/L
GROUP A				
A-1	1201	**25**	72.7	n.d. low
A-2	1201	11.7	47.9	2.6
A-3	671	**23**	**119.5**	1.2
A-4	1100	12.3	54.9	**8.1**
A-5	**153**	16	**145.9**	**7.5**
A-6	691	19.4	76.2	1.8
A-7	994	14.4	56.8	7
A-8	NA	NA	NA	NA
GROUP B				
B-1	**99**	19.5	**134.1**	**9.5**
B-2	**252**	17.8	68.4	5.5
B-3	**99**	15.2	**154.5**	**54.8**
B-4	**99**	10.9	**107.1**	**12.1**
B-5	**153**	17.8	**93.1**	**9.5**
B-6	NA	NA	NA	NA
B-7	**139**	**7.1**	**206.7**	**35**
B-8	**99**	16.6	41.5	4.8
B-9	**287**	16.5	**102.3**	4.1
B-10	**99**	13.6	29.1	6.1
B-11	1201	14	58.7	3.7
B-12	668	13.6	**112.5**	5.9

n.d. low, not determinable low
NA, not available

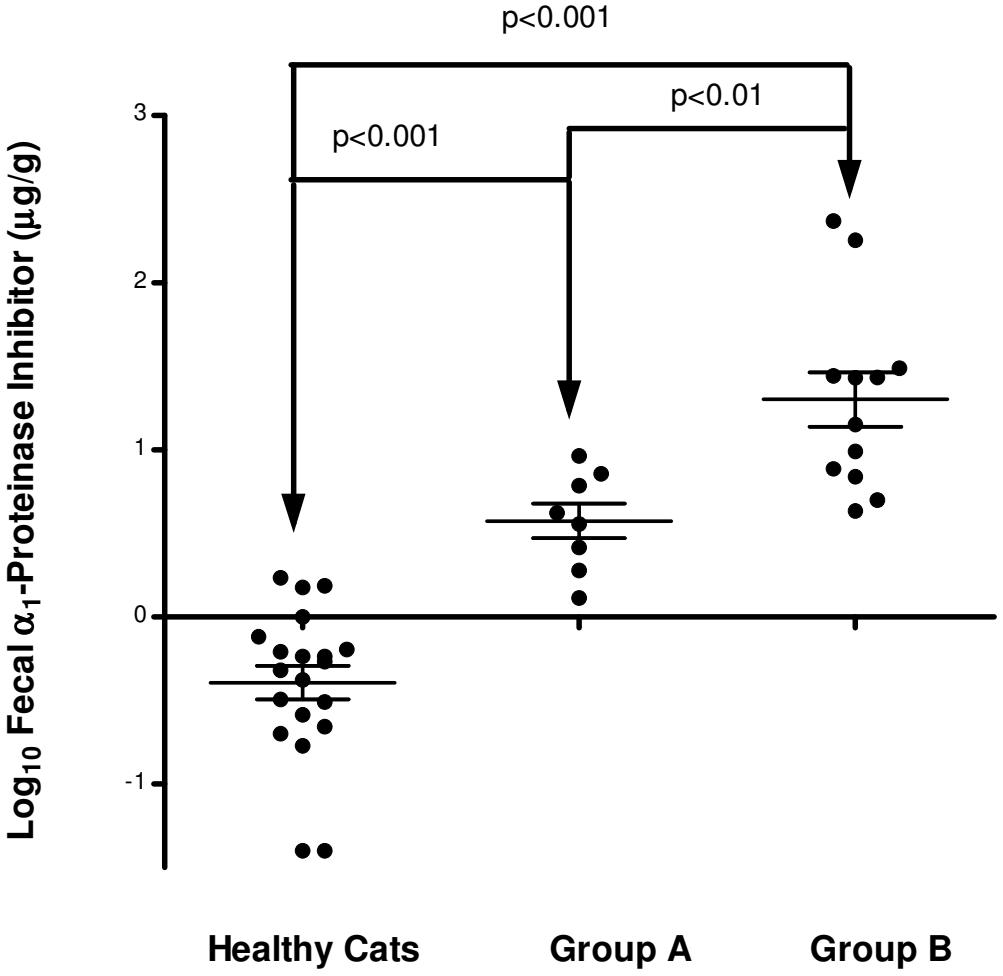

Figure 4. Fecal α_1-PI concentrations in 20 apparently healthy cats and 20 cats with chronic GI disease (Group A: mild to moderate IBD; Group B: severe IBD or gastrointestinal neoplasia). Both cats of Group A (median: 3.9 µg/g, range: 1.3 to 9.2 µg/g) and those of Group B (median: 20.6 µg/g, range: 4.3 to 233.6 µg/g) had statistically significant different fecal α_1-PI concentrations compared to healthy cats (median: 0.51, range: 0.04-1.72), (1-way ANOVA; $P<0.001$, $P<0.001$, respectively). Fecal α_1-PI concentrations were also statistically significant between Groups A and B (1-way ANOVA; $P<0.01$).

DISCUSSION

Our study represents the first evaluation of fecal α_1-PI concentrations in cats with chronic GI disease. We found that, except one cat with low disease severity, cats with gastrointestinal disease had increased fecal α_1-PI concentrations when compared to a group of apparently healthy cats. These data suggest that increased fecal α_1-PI concentrations may be common in cats with IBD and cats with GI neoplasia, such as lymphoma or carcinoma. Furthermore, fecal α_1-PI concentrations were significantly higher in cats with severe IBD or confirmed GI neoplasia (Group B) when compared to cats with mild to moderate IBD (Group A; Figure 4). This is not an unexpected finding, as cases with more severe histopathological lesions (as in cats of Group B in our study) likely have more mucosal disruption and therefore greater transmucosal protein loss. Fecal α_1-PI, which is referred to as α_1-antitrypsin in human medicine, was shown to be increased in human patients with inflammatory bowel disease, such as Crohn's disease, compared to healthy controls (Thomas et al., 1981). Similarly, a previous study in dogs showed that fecal α_1-PI concentrations were significantly higher in dogs with chronic GI disease associated with histological abnormalities compared to dogs with clinical signs of chronic GI disease but normal histology or healthy control dogs (Murphy et al., 2003).

We consider mucosal damage with subsequent loss of plasma as the most likely source of increased α_1-PI concentrations in feces. In humans and various other mammalian species, α_1-PI also has been reported to be produced by intestinal epithelial cells. However, this has not been found to be the case in carnivores (Satoh et al., 1990). Intestinal blood loss (hematochezia or melena) without evidence of GI mucosal disease

also may cause increased fecal α_1-PI concentrations; however one study has shown that even moderate rectal bleeding does not affect fecal α_1-PI determination in human patients (Grill et al., 1984). Although overt blood was not noted on the feces of any of the diseased cats in our study, we cannot completely rule out the possibility of some minor bleeding contributing to the increased concentration of α_1-PI in the feces in some cats.

In the majority of the diseased cats in this study, increased fecal α_1-PI concentrations occurred in association with hypoproteinemia due to hypoalbuminemia. These results suggest that increased fecal α_1-PI concentrations in association with hypoalbuminemia concentrations may be a common finding in cats with IBD and GI neoplasia. In dogs with chronic enteropathies, hypoalbuminemia has further been shown to be a negative prognostic indicator (Allenspach et al., 2007). Hypoalbuminemia has been reported in association with GI inflammation in cats; however, with less frequency (Dennis et al., 1992; Hart et al., 1994; Jergens et al., 1992). Previous studies also have stated that hypoproteinemia is much less common in cats than dogs with GI disease, and that cats appear to develop PLE less frequently than dogs (Lecoindre and Chevallier, 1997; Baez et al., 1999; Peterson and Willard, 2003). In our study, the prevalence of hypoproteinemia was higher in cats of Group B (100%) compared to cats of Group A (57%), suggesting that protein loss via the damaged mucosa occurs more frequently in cases with more severe histological lesions. Panhypoproteinemia is an important indicator of protein-losing enteropathy as opposed to renal or hepatic disease, in which hypoproteinemia is usually caused by hypoalbuminemia alone. In our study,

panhypoproteinemia was present in 38% of all diseased cats, with Group B cats being panhypoproteinemic more frequently (55%) than cats in Group A (14%).

One cat in Group A (A-5) had fecal α_1-PI concentrations within the reference interval while the serum albumin concentration was subnormal. This cat was diagnosed with moderate IBD, and records also revealed concurrent severe chronic cholangiohepatitis and portal hepatitis. The cause of the subnormal serum albumin concentration in this cat remains unclear. It is possible that additional fecal samples from this cat may have shown increased α_1-PI concentrations. We have previously shown that healthy cats had significant variations of fecal α_1-PI concentrations (coefficient of variation: 47%) between samples of three consecutive days (Burke et al., 2012). It appears likely that fecal α_1-PI concentrations may also vary in cats with GI disease, and we therefore prefer a minimum of three samples from consecutive days. Further, insufficient protein synthesis due to hepatitis may have also contributed to the low albumin concentration in this cat. No other cats were diagnosed with overt hepatic or renal disease. Other potential causes for hypoalbuminemia, such as hemorrhage or dermal burns were not reported in any of the cats. Albumin is also a negative acute phase protein, meaning that plasma concentrations can decrease in cases of acute inflammation. In our study, clinical disease of the cats had been observed for several weeks or months, thus it is unlikely that the GI disease of these cats was acute. Given that chronic signs of GI disease and histopathologic lesions were present, and other causes of hypoalbuminemia had been excluded out before enrolling the cats, intestinal

protein loss was the most likely cause for the hypoalbuminemia observed in these cats with increased fecal α_1-PI concentrations.

Hypocobalaminemia has most commonly been reported in cats with severe GI disease or exocrine pancreatic insufficiency (Simpson et al., 2001). Chronic mucosal disease of the ileum may cause destruction or reduced expression of cobalamin-intrinsic factor receptors, which can lead to cobalamin malabsorption and eventually to cobalamin deficiency. Thus, after excluding exocrine pancreatic insufficiency (acinar cells are the source of intrinsic factor in the cat), subnormal serum cobalamin concentrations are highly suggestive of distal small intestinal disease. In our study, hypocobalaminemia was observed in 56% of all cats with chronic GI disease, and was especially frequent in the cats of Group B (81%). Hypocobalaminemia was present in all cats in Group B that were diagnosed with severe GI inflammation or GI lymphoma, diseases commonly involving the ileum. Only two cats in this group had normocobalaminemia, both of which were diagnosed with adenocarcinoma of the colon. None of the cats in our study had decreased serum TLI concentrations, therefore EPI as a cause of hypocobalaminemia was ruled out. Hypocobalaminemia was infrequent in the cats with mild or moderate IBD (Group A, 1/7). It is possible that the lower degree of mucosal disease in these cats did not diminish cobalamin absorption significantly and / or that cobalamin malabsorption had not been present sufficiently long to deplete body stores.

Folate represents a marker for proximal intestinal disease, as this is the location of specific folate carriers. Similar to cobalamin receptors in the distal intestine, folate

carriers in the proximal intestine may be damaged with mucosal disease, thus leading to folate malabsorption, subsequent depletion of folate stores, and, eventually, to folate deficiency. Folate was decreased in only one cat from Group B. This low frequency of decreased folate concentrations may be due to absence or insignificant mucosal damage of the proximal intestine in these cats. Alternatively, malabsorption may have been present, but folate stores were not depleted. Also, serum folate concentration may have been falsely normal or increased in patients with cobalamin deficiency, as cobalamin acts as a cofactor for an enzymatic pathway, which uses folate. Increased serum folate concentrations, as present in one cat from Group A, have no known clinical significance in cats.

Serum fPLI is currently considered to be the most sensitive test for the diagnosis of pancreatitis in cats, being superior to both fTLI and abdominal ultrasound (Forman et al., 2004). Two cats in Group A presented with slightly increased serum fPLI concentrations, both of which were not in the range suggestive for pancreatitis ($\geq$ 12 µg/L). In contrast, five of the cats diagnosed with severe IBD or neoplasia (Group B) had increased serum fPLI concentrations; three of these values were suggestive of pancreatitis. The presence of pancreatitis in some of the cats in our study is not surprising, as coexistence of IBD and pancreatitis has been reported previously (Weiss et al., 1996; Meneses et al., 2003). The three cats with apparent pancreatitis also had concurrent hypoalbuminemia and hypocobalaminemia. A recent study has shown that hypocobalaminemia and hypoalbuminemia were more frequent in cats with IBD that had a concurrently increased serum fPLI concentration (Bailey et al., 2010).

All three cats with serum fPLI concentrations suggestive of pancreatitis also had increased serum fTLI concentrations. Three of the four cats with slightly increased serum fPLI concentrations (6.9 to <12 µg/L) also had increased serum fTLI concentrations. However serum fTLI concentrations were also increased in three cats in which the serum fPLI concentration was within the reference interval. Increases of serum fTLI concentrations in cats with IBD have been reported previously; however the reason for this is not known (Simpson et al., 2001).

There are some limitations associated with our study. First, we were only able to obtain a single fecal sample from each cat for the fecal α_1-PI assay. As mentioned above, we have previously reported significant variations of fecal α_1-PI concentrations between samples of three consecutive days in healthy cats (Burke et al., 2012). We therefore recommend analysis of three fecal samples from consecutive days and calculation of the mean fecal α_1-PI concentration. However, despite only having a single fecal sample available for the current study, we were able to demonstrate that fecal α_1-PI concentration was statistically significant different between the two groups. However, only having a single sample available may have led to the fecal α_1-PI within the normal range identified in one of the hypoalbuminemic cats in Group A.

The number of cats for each group was low. For example, we only were able to enroll two cats with severe IBD. Therefore, we were not able to create a separate group for this category. We decided to place these two cats into Group B, because severe IBD is often difficult to differentiate from neoplastic disease, and because progression of lymphocytic inflammation to lymphoma has been proposed (Wilcock, 1992; Hart et al.,

1994; Krecic, 2001). Finally, histopathological evaluations were performed by several pathologists. A previous study has shown significant inter-observer variation regarding histopathological interpretations of feline and canine intestinal tissues (Willard et al., 2002). In that study, pathologists were from different institutions. In contrast, all pathologists in our study were from the same institution (AMC, NY) and cooperation between pathologists occurred, which likely created a more standardized grading scheme within this institution when compared to the study previously reported.

CHAPTER IV

DISCUSSION

In the first part of our study, we successfully developed and analytically validated an immunoassay for the measurement of α_1-PI in feces and serum of cats. In comparison to the previously developed radioimmunoassay for the measurement of α_1-PI in serum (Fetz et al., 2004), this new ELISA can be utilized to quantify α_1-PI in both serum and fecal samples from cats. Other advantages of the ELISA compared to the RIA include the faster turn-around time (about 6 hours) and the avoidance of radioactive material. Results of the validation of the ELISA demonstrated sufficient linearity, accuracy, precision, and reproducibility for both serum and fecal samples of cats.

Although, α_1-PI has been identified and purified in many different species, assays that can be used to quantify this protein in biological fluids have, thus far, been limited to humans and dogs. The availability of assays usually depends on the clinical need for such an assay. Assays for the measurement of α_1-PI in serum from humans were developed for the identification of α_1-PI deficiency, which is a common condition in people, but has not yet been reported in other species. Alpha$_1$-PI is also an acute phase protein in a few species, including humans, rats, and rabbits. However, other serum markers, e.g., C-reactive protein, are routinely used to monitor the acute phase responses. Its relative abundance in plasma, coupled with its unique property of being resistant to proteolysis, makes α_1-PI a suitable marker for intestinal protein loss. Protein-

losing enteropathies have been associated with many different disease conditions in humans and dogs, while fewer reports exist in cats. The ELISA for feline α_1-PI developed in this study is technically quite similar to the assay previously developed for use in dogs.

In the second part of our study we evaluated fecal α_1-PI concentrations as a marker of gastrointestinal protein loss in cats with chronic gastroenteropathies. Protein-losing enteropathies have been associated with a variety of disease conditions in dogs, including idiopathic inflammatory bowel disease, intestinal lymphoma, and lymphangiectasia. Generally, a diagnosis of PLE can be assumed when hypoalbuminemia is present and other conditions that can cause hypoalbuminemia (e.g., renal loss, hepatic failure, blood loss, or cutaneous lesions) have been excluded. Although hypoproteinemia and hypoalbuminemia have been reported in cats with gastroenteropathies, the term PLE is rarely used in cats. One reason may be that, thus far, intestinal protein loss could not be readily diagnosed in cats due to the lack of a confirmatory test. Another reason may be that hypoalbuminemia appears to be less frequent and less severe in cats compared to dogs with gastrointestinal disease, and therefore may not be regarded as a significant complication in this species. Hypoalbuminemia is usually noted at a time when the disease is long-standing and severe. At that time, protein synthesis by the liver can no longer fully compensate for the continuous intestinal loss of protein. The quantification of α_1-PI in fecal matter allows the detection and quantification of intestinal protein loss. This method therefore has the

potential to identify PLE much earlier in the disease process and likely before hypoalbuminemia or severe clinical signs are present.

We showed that increased fecal α_1-PI concentrations were present in the majority of the diseased cats compared to healthy controls. Our study, therefore, provides direct evidence that intestinal protein loss also does occur in cats with chronic gastrointestinal disease. The study design should be regarded as a pilot study as we could only enroll a relatively low number of healthy and diseased cats. In addition, only a single fecal sample was available from the diseased cats, while we generally recommend the collection of three fecal samples from consecutive days. Interestingly, we also found that fecal α_1-PI concentrations were higher in cats with greater severity of histopathological lesions. This finding was not unexpected as more transmucosal protein exudation can be expected with more severe histopathological lesions. Similar findings have also been reported in a study of dogs with chronic gastrointestinal disease (Murphy et al., 2003).

Comparison of fecal excretion of α_1-PI with that of radioactively labeled leukocytes correlated well in one study in humans (Fischbach et al., 1987), but not in another (Crama-Bohbouth, 1989). This suggests that gastrointestinal protein loss does not only depend on mucosal inflammation, and that gastrointestinal protein loss is not always a marker of mucosal inflammation. Fecal biomarkers for intestinal inflammation, such as calprotectin (S100A8/A9) and S100A12 recently have become available in dogs (Heilmann et al., 2008, Heilmann et al., 2011). Should these assays become available for

use in cats, it would be interesting to compare these fecal inflammatory markers with fecal α_1-PI concentration in cats with chronic GI disease.

Hypoproteinemia and hypoalbuminemia have previously been associated with IBD and intestinal lymphoma in cats. For example, Jergens et al. (1992) found hypoalbuminemia in 24% of cats with IBD. Another study by Baez at al. (1999) reported hypoproteinemia and hypoalbuminemia in 24% and 15% of cats with IBD, respectively. Mahony et al. (1995) found mild hypoalbuminemia in 23% of cats with intestinal lymphoma, while low-grade intestinal lymphoma was not associated with hypoalbuminemia in a study by Lingard et al. (2009). Compared to these previous studies, hypoalbuminemia and hypoproteinemia were observed much more frequently in the cats with IBD (71% and 57%, respectively) or cats with neoplasia (100% and 100%, respectively) in our study. It is possible that the cats with IBD in our study had disease of a more pronounced severity compared to cats of other studies. Further, overall neoplastic disease in the cats of our study was possibly in more advanced stages than in previous studies, which may explain the higher prevalence of hypoalbuminemia and hypoproteinemia.

CHAPTER V

CONCLUSIONS

We conclude that the ELISA for the measurement of feline α_1-PI concentrations in serum and fecal samples from cats described here is sufficiently linear, accurate, precise, and reproducible.

Further, this is the first study investigating fecal α_1-PI concentrations as a marker of GI protein loss in cats with IBD or GI neoplasia. We showed that increased fecal α_1-PI concentrations were present in the vast majority of the diseased cats compared to healthy controls and that fecal α_1-PI concentrations were higher in cats with greater severity of histopathological lesions. The frequent presence of hypoalbuminemia in these cats further suggests a protein-losing syndrome as it has been described in humans and dogs with chronic gastroenteropathies before. Further studies with larger groups of healthy and diseased cats are warranted for the clinical evaluation of this assay.

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