Universitat Autònoma de Barcelona

P-glycoprotein, Multidrug Resistance 1 gene, and Cyclooxygenase 2 expression in cats with Inflammatory Bowel Disease and Low Grade Alimentary Lymphoma

Jorge Castro López

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Universitat Autònoma de Barcelona

PhD Thesis

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"P-glycoprotein, Multidrug Resistance 1 gene, and Cyclooxygenase 2 expression in cats with Inflammatory Bowel Disease and Low Grade Alimentary Lymphoma"

Presented by: **Jorge Castro López**

In order to obtain the degree of PhD in Animal Medicine and Health

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Los Doctores JOSEP PASTOR MILÁN, MARTA PLANELLAS BACHS y MARIANA TELES, Profesor Titular y Asociado del Departamento de Medicina y Cirugía Animal de la Facultad de Veterinaria de la Univesitat Autònoma de Barcelona, e Investigadora de, respectivamente.

INFORMAN:

Que la memoria titulada "Detección de la expresión de la glicoproteína P, gen de resistencia a multidrogas tipo 1 (multidrug resistance-1 gene), y ciclooxigenasa 2 en gatos con enfermedad inflamatoria intestinal y linfoma de grado bajo", presentada por el licenciado en veterinaria JORGE CASTRO LÓPEZ para la obtención del grado de Doctor en Veterinaria, se ha realizado bajo nuestra dirección y, considerándola satisfactoriamente finalizada, autorizamos su presentación para que sea evaluada por la comisión correspondiente.

Y para que así conste a los efectos que sean oportunos, firmamos el presente informe en Bellaterra, 28 de septiembre de 2017.

Director: **Josep Pastor Milán** Directora: **Marta Planellas Bachs** Directora: **Mariana Teles**

"Queda prohibido no sonreír a los problemas, no luchar por lo que quieres, abandonarlo todo por miedo, no convertir en realidad tus sueños…"

Pablo Neruda

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1. INTRODUCTION

Inflammatory bowel disease (IBD) refers to a group of idiopathic chronic gastrointestinal (GI) tract disorders characterized by persistent or recurrent clinical signs and inflammation of the GI tract (Jergens, 2012). This inflammation can affect other organs, mainly liver and pancreas what is called triaditis (small intestine, liver and pancreas), but kidneys involvement has been reported (Simpson, 2015). The aetiology of this disease remains unknown and likely is multifactorial mediated by an inappropriate and uncontrolled inflammation of intestinal mucosal-associated lymphoid tissue against harmless environmental antigens present in the diet or commensal bacteria (Allenspach, 2011).

IBD was first reported in small animals around 25 years ago and, nowadays, it is one of the most common diagnoses in cats and dogs with chronic enteropathy (CE) (Washabau et al., 2010). Currently, the diagnosis is made by exclusion, because it is necessary to rule out extraGI and other GI causes. This is performed using antiparasitic, exclusion diet (novel or hydrolysed protein), and mainly in dogs, antibiotic such as metronidazole and tylosin. Afterward, biopsies are obtained by endoscopy or laparotomy, however another less used technique is laparoscopy. The World Small Animal Veterinary Association (WSAVA) International GI Standardization Group has published guidelines for histological interpretation and standardization. Furthermore, the American College of Veterinary Internal Medicine also published guidelines to interpret various diagnostic test, including treatment trials, patient response, and

outcome (Washabau et al., 2010). Both statements mentioned that IBD never should be diagnosed based on histopathology alone and that biopsy samples are only one part of a comprehensive workup (Day et al., 2008; Washabau et al., 2010).

Lymphoma is the most common type of haematopoietic neoplasia in cats that can be associated with retrovirus infection (Louwerens et al., 2005). However, most of cats with alimentary lymphoma (AL) are negative for feline leukemia virus and immunodeficiency virus infection (Louwerens et al., Barr and Beatty, 2012a). Other risk factors are chronic inflammation, and exposure to cigarette smoke (Barr and Beatty, 2012a). Helicobacter infection may also play a role in the pathogenesis of the disease but further studies are needed to confirm it (Bridgeford et al., 2008). The current most common form of lymphoma in cat is the GI one that have increased over time (Barr and Beatty, 2012). The AL is commonly classified as low grade (LG), intermediate grade (IG) and high grade (HG) form (Valli et al., 2000; Valli et al., 2011). There is another form called large granular lymphocyte lymphoma that is consider apart from the other 3 forms mentioned above. In this project, we are going to focus on the LGAL.

IBD and LGAL are very similar clinically and histologically. The clinical history of chronic GI signs and findings from the physical examination, hematology, biochemistry, specific tests such as folate and cobalamin, abdominal ultrasound, and even histopathologic changes of IBD and LGAL often overlap (Jergens, 2012; Barrs and Beatty, 2012a; Barrs and Beatty, 2012b). Therefore, differentiating between both

diseases may be a challenge; immunohistochemistry and polymerase chain reaction (PCR) for antigen receptor rearrangement are diagnostic tools for obtaining a more accurate diagnosis (Kiupel et al., 2011).

Permeability glycoprotein (P-gp) is a synthetized by Multidrug resistance 1 (*MDR-1*) gene that might be implicated in susceptibility and pathogenesis, severity of the disease and outcome of IBD and lymphoma (Bergman et al., 1996; Ginn et al., 1996; Lee et al., 1996; Sandor et al., 1997; Ho et al., 2003; Fromm, 2002; Buyse et al., 2005; Wilk et al., 2005; Allenspach et al., 2006; Brenn et al., 2008; Van der Heyden et al., 2011b). Difference between species has been reported in healthy and sick individuals about epithelial and lamina propria expression in the IBD as well as lymphoma (Cordon-Cardo et al., 1990; Bergman et al., 1996; Ginn et al., 1996; Buyse et al., 2005; Wilk et al., 2005; Brenn et al., 2008; Van der Heyden et al., 2009; Van der Heyden et al., 2011b).

Cyclooxygenase 2 (COX-2) is an inducible inflammatory regulator isoform by cellular activation, proinflammatory cytokines, growth factors, tumour promoters and prostaglandin mediator (Vane et al., 1998; Williams et al., 1999; Yu et al., 2007; Ghosh et al., 2010). COX-2 has been described in the intestine of healthy individual of several species (Beam et al., 2003; Satoh et al., 2013; Jackson et al., 2000; Paiotti et al., 2007; Romero et al.,2008; Dai, 2015). Furthermore, higher COX-2 expression has been

reported in human and canine IBD, and lymphoma (Singer et al., 1998; Jackson et al, 2000; Ohsawa et al., 2006; Østergaard et al., 2009; Dumusc et al., 2014).

Correlation between COX-2 and P-gp immunoexpression has been reported in hepatocellular cancer cells, breast cancer cells and ovarian cancer cells in human beings but not in IBD and lymphoma (Fantappie et al., 2002; Surowiak et al., 2005; Surowiak et al., 2006).

In the present project, studies about P-gp, *MDR-1* gene and COX-2 expression by immunohistochemistry and PCR were carried out in cats with IBD and LGAL.

2. AIMS

The major aims of this project were:

- Investigate P-gp immunoexpression in the epithelium and lamina propria of small intestine of cats with IBD and LGAL.
- Investigate COX-2 immunoexpression in the epithelium and lamina propria of cats with IBD and LGAL.
- Determine the mRNA transcription levels of *MDR-1* and *COX-2* in cats with IBD and LGAL.

The secondary aims of this project were:

- Correlate P-gp, COX-2 and their genes with clinical signs and histological severity.
- Evaluate a correlation between *Mdr-1* and *Cox-2* genes.

3. LITERATURE REVIEW

3.1 AETHIOPATHOGENESIS

3.1.1 INFLAMMATORY BOWEL DISEASE (IBD)

IBD refers to a group of idiopathic chronic gastrointestinal (GI) tract disorders that are immunologically mediated and characterized by persistent or recurrent GI clinical signs and histologic inflammation (Jergens, 2012; Day et al., 2008). The aetiology is multifactorial and involves complex interactions between genetics, the mucosal immune system and environmental factors. Enteric microbiota imbalances (dysbiosis) and dietary components are recognized as antigens by the gut-associated lymphoid tissue from a genetically susceptible cat resulting in aberrant, inappropriate and uncontrolled chronic inflammation. Environmental factors such as dietary constituents, exposure to enteropathogens, non-steroidal anti-inflammatory drug or antibiotic administration likely govern inflammation onset or reactivation (Figure 1) (Allenspach, 2011; Jergens, 2012).

Genetic defects in the recognition of commensal versus pathogenic bacteria by the innate immune system play a pivotal role in disease pathogenesis in humans and dogs with IBD. Therefore, it is believed that the innate immune system reacts to normal commensals in the intestinal lumen as if they were pathogens (Allenspach, 2011; Jergens, 2012). Mutations or down/upregulation of innate immune receptors of humans [Toll-like receptor (TLR) 2, TLR4, nucleotide-binding oligomerization (NOD) 2/ caspase recruitment domain-containing protein 15 (CARD15)] and dogs (TLR2, TLR4,

TLR5, TLR9, NOD2) have now been linked to IBD susceptibility (Jergens, 2012; Xavier and Podolsky, 2007; Kathrani et al., 2012, Kathrani et al., 2014; Honneffer et al., 2014). However, innate immune receptors implication in feline IBD are currently unknown.

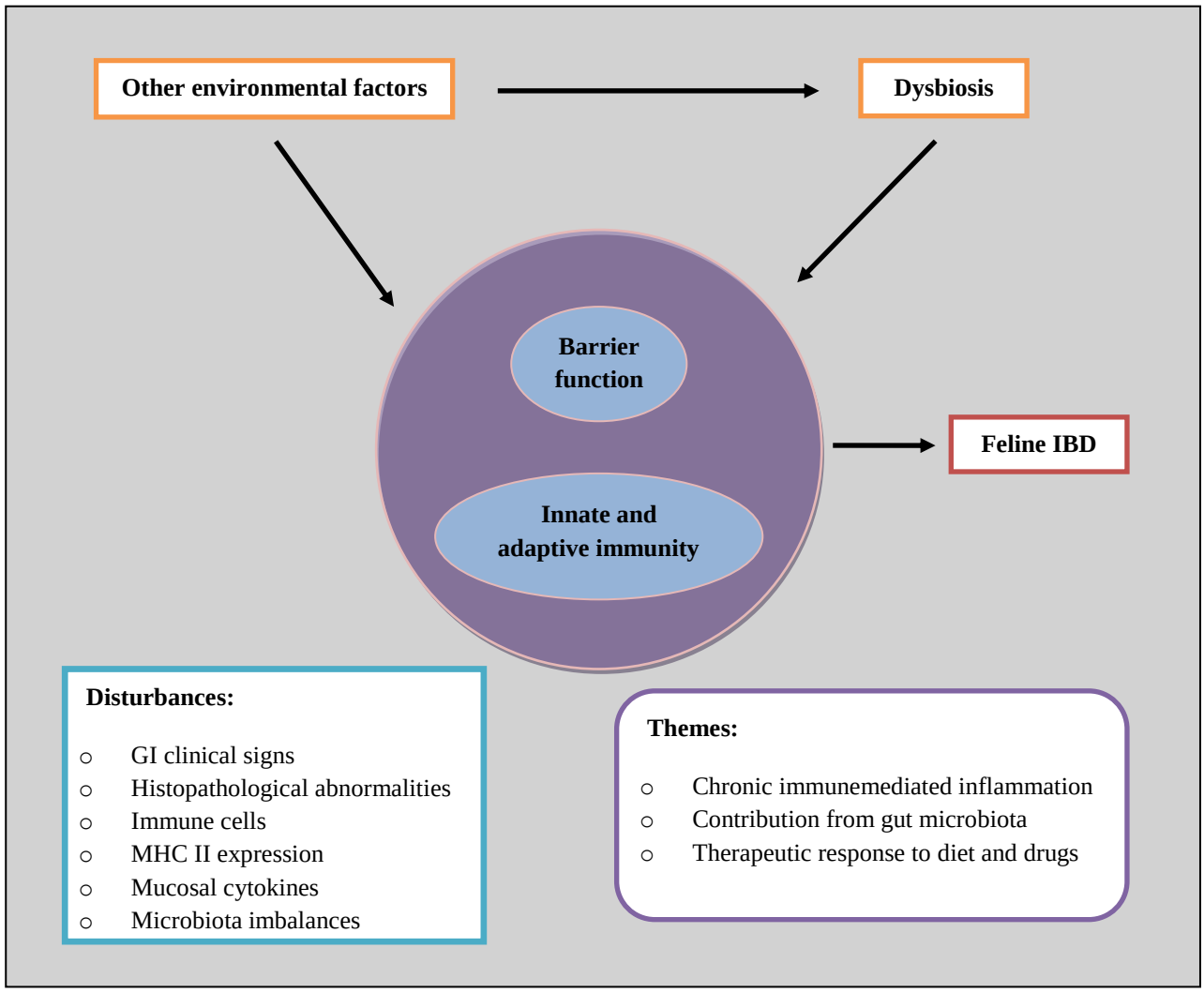

Figure 1. Proposed pathogenesis of feline IBD (Modified from Jergens 2012).

The presence of an enteric microbiota may lead to upregulated proinflammatory cytokine production and reduced bacterial clearance, thereby promoting chronic intestinal inflammation (Jergens, 2012; Honneffer et al., 2014). Scarce published information is available about the mucosa-adherent microbiota of cats with IBD. One study reported an increase in *Enterobacteriaceae* in duodenal biopsies of cats with IBD and recently *Campylobacter coli* association has been reported in neutrophilic IBD in cats (Janeczko et al., 2008; Mauder et al., 2016). Another report in cats with IBD described lower counts for total bacteria (*Bacteroides*, *Bifidobacterium*) but higher counts of *Desulfovibrio* compared to healthy cats that could be associated with the feline IBD pathogenesis (Inness et al., 2007). However, these counts were not confirmed in another study therefore conclusions cannot be obtained (Abecia et al., 2010). In a recent pyrosequencing study, several bacterial groups correlated with improved foecal scores after therapeutic response to diet, those included *Slackia* spp., *Campylobacter upsaliensis*, *Enterobacteriaceae Raoultella* spp., *Collinsella* spp., and unidentified genera within *Clostridiales* and *Lachnospiraceae* but it could not be determined whether changes in the microbiota caused a direct improvement in diarrhoea, or vice versa (Ramadan et al., 2014).

Microbiota may be implicated in the induction of cytokines production. Only a few studies have investigated about cytokine profile in feline IBD. One report described, based on histologic changes, that cats with intestinal inflammation had

significantly more transcription of genes encoding interleukin (IL)-6, IL-10, IL-12p40, tumour necrosis factor alpha and tissue growth factor beta than those with normal intestinal morphology (Nguyen et al., 2006). Janeczko et al. (2008) described that the number of *Enterobacteriaceae*, *E. coli*, and *Clostridium* spp. correlated with upregulation of IL-1, IL-8 and IL-12 mRNA, abnormalities in mucosal architecture and the number of clinical signs exhibited by the affected cats. Recently, it has been reported that cats with IBD presented IL-23 upregulation and IL-12p35 downregulation compared to healthy cats and no difference in expression of IL-12p40 and interferon-γ mRNA were observed (Waly et al., 2014). In humans being, IL-23 has been implicated in the IBD pathogenesis as well as may be in cats according to this last study.

Another study reported upregulated epithelial major histocompatibility complex (MHC) class II expression by cells with dendritic morphology in the lamina propria, and increased IgM reactivity from plasma cells that may reflect again an alteration in duodenal bacterial flora, which may be involved in the immunopathogenesis of feline IBD (Waly et al., 2004).

A genetic basis for IBD in some cat breeds is suspected though causal genetic defects have not been identified to date. Future studies are needed to identify possible genetic predispositions in certain feline breeds that contribute to IBD development.

3.1.2 FELINE ALIMENTARY LYMPHOMA

Currently, alimentary lymphoma (AL) is the most common anatomical form identified (Mahony et al., 1995; Gabor et al., 1998; Vail et al., 1998; Louwerens et al., 2005; Milner et al., 2005; Stutzer et al., 2011; Vezzali et al., 2010; Weiss et al., 2010). Additionally, lymphoma is the most common intestinal neoplasm described in cats (55%), followed by adenocarcinoma (32%) and then mast cell tumour (4%) according to Risetto et al. (2011) that studied 1129 feline intestinal neoplasms.

Several risk factors have been associated with feline lymphoma. One of the most important risks was the feline leukemia virus (FeLV) but in the current post-FeLV era a significant decrease in the importance of FeLV-associated types of lymphoma in cats has been reported (Louwerens et al., 2005). AL shows the lowest association with FeLV antigenaemia generally being detected in 0 to 12% of cases (Mahony et al., 1995; Vail et al., 1998; Stutzer et al., 2011; Zwahlen et al., 1998; Rassnick et al., 1999; Fondacaro et al., 1999). Currently, the detection of FeLV provirus deoxyribonucleic acid (DNA) technique is available and it has been proposed that provirus may be implicated on tumourgenesis in antigen-negative cats. Weiss et al. (2010) described that 80% of T cell lymphomas and 60% of B cell lymphomas contained provirus DNA while only 21% of T-cell lymphomas and 11% of B cell lymphomas expressed FeLV antigen and likely provirus DNA is implicated. Opposite to this, Stützer et al (2011) reported that none of the antigen-negative cats with lymphoma was detectably infected with latent FeLV and

concluded that latent FeLV infection is unlikely to be responsible for most feline lymphomas. This difference between both studies likely is because of the different PCR technique used and design of the study, since in the second one FeLV antiginaemia was performed in serum of all cats recruited and negative provirus DNA results correlated with no FeLV p27 immunohistochemistry expression in the tumour.

Feline immunodeficiency virus (FIV) is another risk factor that might have an indirect role in lymphomagenesis (Barrs and Beatty, 2012). One study that described 4.3% of cats FIV positive into a population, 21% of them presented lymphoma being AL the most common (Feder and Hurvitz, 1990). However, in more recent studies no association is reported.

Helicobacter infection may play a role in the development of feline gastric lymphoma (Bridgeford et al., 2008). In this study, gastric biopsy samples from 16 of 24 cats with lymphoma were positive for *Helicobacter heilmannii*. The potential importance of this infection is that eradication of the bacteria with antibiotics may resolve or hinder the progression of the underlying neoplasm is still unknown.

Other risk factors are tobacco smoke and chronic inflammation. Cats living in households with any exposure to cigarette smoke have a 2.4-fold increased risk of developing lymphoma than cats from nonsmoking households, and the amount and duration of exposure is linearly correlated with increasing risk of lymphoma

development since cats living more than 5 years exposure have 3.2-fold increased risk (Bertone et al., 2002). Otherwise, enteropathy-associated T cell lymphoma can arise from a chronic antigenic stimulation in coeliac people (Woodward, 2016). In cats, some studies suggest that chronic inflammation may also progress to lymphoma. In two studies of lymphoma, 60% of cats with intestinal T cell lymphoma and 33% of cats with large granular lymphocyte lymphoma (LGLL) had chronic clinical illnesses suggestive of pre-existing inflammatory disease (Moore et al., 2005; Roccabianca et al., 2006). Nevertheless, concurrent lymphoplasmacytic enteritis (LPE) has been identified in other regions of the alimentary tract in up to 41% of cats with LGAL (Carreras et al., 2003; Lingard et al., 2009; Briscoe et al., 2011; Scott et al., 2011). Therefore, both diseases may coexist in the same individual. More evidence is needed to draw that chronic inflammation may progress to AL in cats.

3.2 SIGNALMENT

IBD affects middle-aged cats but it has been reported in younger as 2 years (Jergens et al., 1992). Certain predisposition has been proposed for Siamese and Oriental breeds but any cats can be affected (Jergens, 2012). On the other hand, AL affects middle to old aged cats, with a median age at diagnosis of 10 to 13 years, however cats between 1 to 20 years have been reported (Barrs and Beatty, 2012a; Mahony et al., 1995; Zwahle et al., 1998; Fondacaro et al., 1999). Male predisposition

has been identified in cats with high grade AL (HGAL, 1.5:1 male to female ratio) in some reports but not for low grade AL (LGAL) (Barrs and Beatty, 2012a; Mahony et al., 1995; Zwahle et al., 1998; Fondacaro et al., 1999). On the other hand, LGLL has been most frequently reported in females in two studies (Endo et al., 1998; Roccabianca et al., 2006). No breed predisposition has been described in any type of feline AL but Domestic Shorthair has been more reported likely due to population overrepresentation.

3.3 CLINICAL PRESENTATION AND PHYSICAL FINDINGS

Clinical signs are similar for both diseases that include weight loss, vomiting, diarrhoea, anorexia, some cases polyphagia, and lethargy (Al-Ghazlat et al., 2013) (Figures 2A and B).

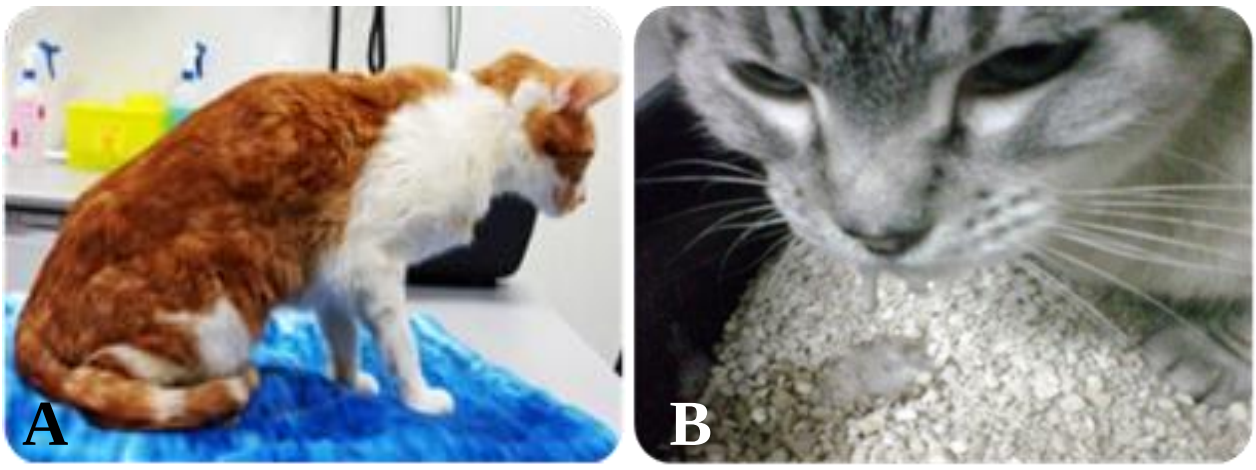

Figure 2. (A) Cat with LGAL and low body condition score. **(B)** Cat with IBD vomiting.

3.3.1 IBD

Vomiting and diarrhoea are common, followed by decreased appetite and weight loss. Gastric and duodenal inflammation is frequently associated with vomiting, whereas jejunum and ileum may produce vomiting and small bowel diarrhoea, and less frequently melaena. Otherwise, colon implication causes large bowel diarrhoea, haematoquezia, mucus, and straining.

In cases of food responsive enteritis (FRE), concurrent dermatologic and GI signs have been reported in about 10 to 15% of food-allergic cats (Muller et al., 2001).

Some cats can present triaditis that involves concurrent inflammation of intestine, pancreas and liver, that also contribute to the clinical sings. A recent prospective study described that cholangitis (22.2%) is more commonly associated with IBD than pancreatitis (7.4%) with a total of 29.6% of cats with clinical signs that presented concomitant inflammation in two organs as well as those cats affected by triaditis (29.6%) (Fragkou et al., 2016). Previous retrospective studies mainly based on necropsy findings reported that cholangitis was associated with IBD 46 to 83% of cats and triaditis was presented in 32 to 50% of cases (Weiss et al., 1996; Callahan Clark et al., 2011; Twedt et al. 2014). Otherwise, studies were the main disease was pancreatitis, IBD was associated between 61 to 65% of cats and triaditis was found in 50 to 56% of cases (Swift et al., 2000; Forman et al., 2004). However, these different studies are not

comparable due to different population, methodology of diagnosis and histological classification. Likely, the most reliable results are from the most recent study where triaditis and concurrent inflammation of two organs are not very common.

The clinical course of IBD is generally cyclical and is characterized by spontaneous exacerbations and remissions (Jergens et al., 2012). Triggers for recurring signs are rarely identified but may include dietary indiscretion, transient exposure to intestinal pathogens or drug administration such as steroids, non-steroidal anti-inflammatory drugs, and antibiotics (Jergens, 1999).

Recently, a feline clinical activity has been proposed by Jergens et al. (2010) similar to humans and dogs (Best et al., 1976; Harvey and Bradshaw, 1980; Jergens et al., 2003; Allenspach et al., 2007). The feline chronic enteropathy activity index (FCEAI) is composed by GI signs (vomiting, diarrhoea, anorexia, weight loss and lethargy), laboratory findings (total protein, phosphorus, ALT and ALP) and endoscopic lesions (granularity, friability, ulcer/erosions), each parameter receives a score. The FCEAI is showed in the Table 1. This index serves to evaluate objectively the response to treatment comparing the initial score with the post-treatment one and to take decision according to the patient's need. Endoscopic lesions parameter may be omitted when repeat in not possible. This index has utility in cats with IBD and FRE (Jergens et al., 2010). The present author evaluated the utility of the FCEAI in 9 cats with IBD, median

score pre-treatment was 11 and 30 days later during the treatment was 5 confirming the easy way to assess the clinical response to treatment (Castro-López et al. 2011).

Table 1. Parameters and scores used for the calculation of the feline chronic enteropathy activity index (FCEAI).

PARAMETERS	**SCORES**				**SUBTOTAL**
GI signs	**0**	**1**	**2**	**3**	
Attitude/activity	Normal	Slightly decreased	Moderately decreased	Severely decreased	
Appetite	Normal	Slightly decreased	Moderately decreased	Severely decreased	
Vomiting	None	Mild (1X/wk)	Moderate (2-3X/wk)	Severe (>3X/wk)	
Diarrhoea	None	Slightly soft feces or blood/mucus or slightly increased (2-3X/d)	Very soft feces or moderately increased (4-5X/d)	Watery diarrhea or severely increased (>5X/d)	
Weight loss	None	Mild (<5%)	Moderate (5-10%)	Severe (>10%)	
Total protein	No	Yes			
ALT/ALP	Normal	Increased			
Phosphorus	Normal	Increased			
Endoscopic lesions	No	Yes			
					TOTAL SCORE

GI: gastrointestinal; ALT: alanino aminotransferase; ALP: alkaline phosphatase

3.3.2 LGAL

The clinical signs are the same that IBD, therefore they are unspecific, and usually are also chronic (weeks to months) (Carreras et al., 2003; Evans et al., 2006; Lingard et al., 2009). The most common clinical signs of LGAL are weight loss (≥ 80%), vomiting (≥ 70%), diarrhoea (≥ 60%) and partial or complete anorexia (≥ 50%), however the appetite may be normal or polyphagia may be present. Less frequently reported signs include lethargy and polydipsia (Fondaraco et al., 1999; Carreras et al., 2003; Evans et al., 2006; Kiselow et al., 2008; Lingard et al., 2009). On the physical examination, diffusely thickened intestinal loops are detected on the abdominal palpation of affected cats (≥ 30%). An abdominal mass is palpable in 20 to 30% of cases, attributable to a mesenteric lymph node enlargement or, rarely, focal intestinal mass (Fondaraco et al., 1999; Carreras et al., 2003; Lingard et al., 2009).

3.4 DIFFERENTIAL DIAGNOSIS

The presenting signs are common for IBD and LGAL and to many primary and secondary GI diseases (Table 2). LGAL is a major differential diagnosis for IBD. In cats with intestinal mural mass lesions, lymphoma, epithelial and mast cell neoplasia are major differentials. Diagnostic tests are recommended to rule out other primary and secondary GI diseases.

Table 2. Extra-gastrointestinal and gastrointestinal causes of chronic vomiting and/or diarrhoea.

Extragastrointestinal causes	
Pancreatic diseases	Chronic pancreatitis Exocrine pancreatic insufficiency Pancreatic carcinoma
Liver diseases	Chronic cholangitis Cirrhosis Extra-intrahepatic colestasis
Endocrine diseases	Hyperthyroidism Hypoadrenocorticism
Renal diseases	Uremia
Systemic diseases	Virus: FIP, FeLV, FIV, FCV Fungus: Histoplasma, *Cryptococcus* spp, Zygomycosis, Pythyosis
Miscellaneous	Hypercalcaemia, pyometra, congestive heart failure, immune-mediated diseases, neoplastic metastasis, toxins and drugs
Gastrointestinal causes	
Parasites	Roundworns, Hookworns
Protozoans	*Giardia* spp., *Tritrichomonas foetus*, *Isospora* spp., *Cryptosporidium*
Bacteria	*Clostridium* spp., *Campylobacter*, *Salmonella*, *E. coli*, *Enterobacter*, *Enterococcus*, SIBO?
Virus	Coronavirus, Torovirus, Rotavirus
Neoplasms	Mast cell tumour, carcinoma, gastrointestinal stromal tumour, haemangiosarcoma, leiomyosarcoma
Miscellaneous	Feline gastrointestinal eosinophilic sclerosing fibroplasias, non-obstructive foreign body, intestinal stenosis, chronic intussusception

3.5 DIAGNOSTIC APPROACH

The approach depends on clinical status of the patient, while the cat is stable it is appropriate do the rule-out protocol (Table 3). Afterward, biopsies can be obtained by endoscopy or surgery (laparatomy or laparoscopy).

In case that the patient is affected by severe clinical signs and compromised status indicated by physical examination and diagnostic tools, the protocol mentioned above may be avoided and biopsies may be obtained immediately.

3.5.1 Clinicopathological abnormalities

Clinicopathological alterations found in cats with IBD or LGAL are unspecific. One study described that more frequent abnormalities detected in feline IBD are hyperproteinaemia, hypophosphataemia and increased ALT and/or ALP (associated with triaditis), though hypoproteinaemia may also be observed but unfrequently (Jergens et al., 2010). The cause of hypophosphataemia is unknown but it might be attributable to malnourishment, malabsorptive disorders, and chronic vomiting or some combination of these aetiologies (Jergens et al., 2010). In feline AL, the most common clinical-pathology alterations are anaemia, neutrophilia, hypoproteinaemia, hypoalbuminaemia and increased liver enzymes when liver is also affected (Barr and

Beatty, 2012). In LGLL, lymphocytosis with LGL morphology may be observed in the 80% of the cases (Roccabianca et al., 2006; Krick et al., 2008; Finotello et al., 2017).

Table 3. Diagnostic investigation for chronic enteropathies in cats

Blood and urinary tests	
Complete blood count	**Cobalamin and folate**
Serum biochemistry	**FIV and FeLV determination**
Total T4	**Urinalysis**
Specific Feline Pancreatic Lipase	
Faecal tests	
Small bowel diarrhoea	Faecal flotation Faecal immunoassays/PCR: *Giardia spp.*, *Cryptosporidium spp.*, *Campylobacter spp.*, Enteropathogenic bacterial toxins
Large bowel diarrhoea	Faecal smear/culture/PCR: Tritrichomonas foetus
Bloody diarrhoea in cat with fever and inflammatory leukogram	Faecal culture: *Salmonella spp.*, *Clostridium spp.*, *Campylobacter spp.*
Diagnostic imaging	
Abdominal ultrasound	Thoracic radiographies
Therapeutic trials	
Fenbendazole (50 mg/kg PO q24h for 5 days)	
Single novel protein and carbohydrate	Hydrolysed protein diet

Hypocobalanaemia is observed more frequently in cats with LGAL than IBD likely is located commonly in the ileum and jejunum where cobalamin is absorbed (Barr and Beatty, 2012a; Maunder et al., 2012). One study reported that 80% of cats with LGAL presented with hypocobalanaemia and folate was low in 4% and high in 37% of cats (Kiselow et al., 2008). Furthermore, some cats with LGAL and IBD can present hypocobalaneamia instead of normal abdominal ultrasound (Jugan and August, 2017). Feline pancreatic lipase immunoreactivity (PLI) may be mild to moderate increased in both diseases due to triaditis or lymphoma spreading (Jergens, 2012; Barr and Beatty, 2012). In cats with triaditis, increased PLI would not be associated with a negative outcome (Bailey et al., 2010).

A recent study has described that cats with IBD and LGAL presents hypovitomisosis D and whose pathophysiology is unclear but it is suspected to be secondary to the anorexia, decreased intestinal absorption, and increased intestinal loss of vitamin D (Lalor et al., 2014). Another research has reported that cats with chronic colitis tended to show lower levels of taurine that might be caused by alterations in the microbiota (Kathrani et al., 2017). Nowadays, these both determinations have only research interest.

Serum lactate dehydrogenase is a negative prognostic factor in cats with lymphoma, however, no difference was observed between IBD and LGAL (Terragni et

al., 2016). Fecal α1-proteinase inhibitor concentrations has been determined in cats with IBD and GI neoplasia. Cats with severe IBD and LGAL showed higher levels of α1-proteinase inhibitor compared to cats with mild to moderate IBD (Burke et al., 2013).

3.5.2 Imaging

Abdominal ultrasonography is superior compared to radiology for the diagnosis of GI tract diseases because abdominal radiographs mainly detect organomegaly or intestinal obstruction (Jergens, 2012). Ultrasound findings may be consistent with normality in some cases of IBD and LGAL (Gaschen, 2011; Jugan and August, 2017). Both IBD and LGAL are characterized by diffuse or segmental distribution in the small intestine, with ultrasonographic features of bowel wall thickening owing to increase of the muscularis propria and preservation of wall layers without or with mass formation (Gaschen, 2011; Daniaux et al., 2014). A recent study found that the ratio of the width of the muscularis to submucosa is usually <1 in unaffected cats, but usually >1 in segments with lymphoma or IBD (Daniaux et al., 2014). This thickening may be due to tumour cell infiltration in LGAL but it also described a hypertrophy of this layer of unknown origin in IBD and LGAL (Daniaux et al., 2014). These results are similar to a previous study that showed older cats with muscularis layer thickening are more likely to have T cell lymphoma than IBD (Zwingenberger et al., 2010) (Figure 3). Furthermore, mesenteric lymphadenopathy may be associated with lymphoma or IBD

but when colic lymphadenomegaly and diffuse thickening of the muscularis layer of the small intestine are observed may be associated with LGAL (Zwingenberger et al., 2010; Daniaux et al., 2014). Other findings that suggest a neoplastic process and that should increase the indications for biopsy include loss of normal wall layering, disproportionally thick muscularis propria, focal intestinal mass, and ascites (Zwingenberger et al., 2010; Gaschen, 2011). Liver and pancreas must be evaluated to detect the presence of triaditis or tumour spreading (Figure 4).

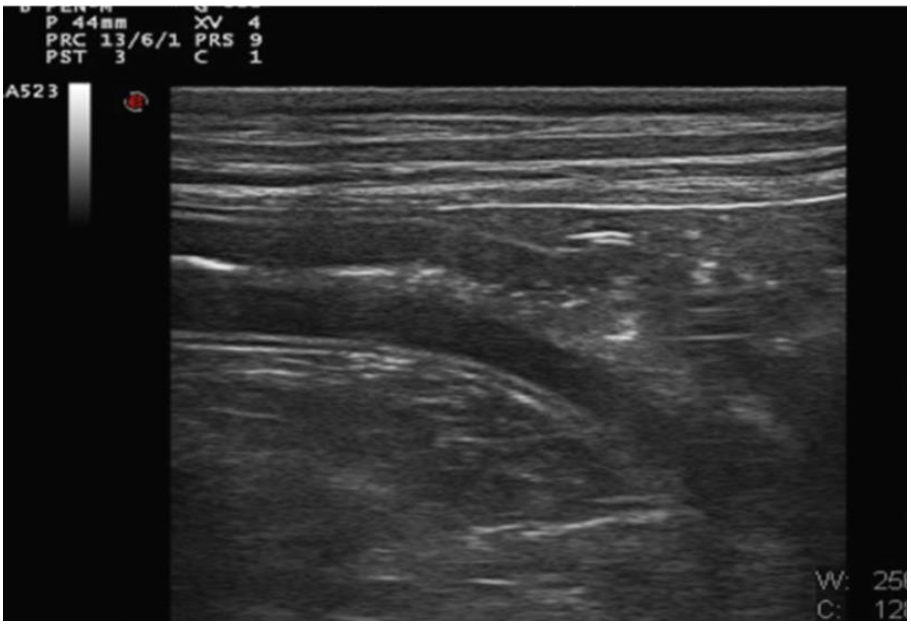

Figure 3. Thickened muscularis wall observed in the abdominal ultrasound in a cat with LGAL.

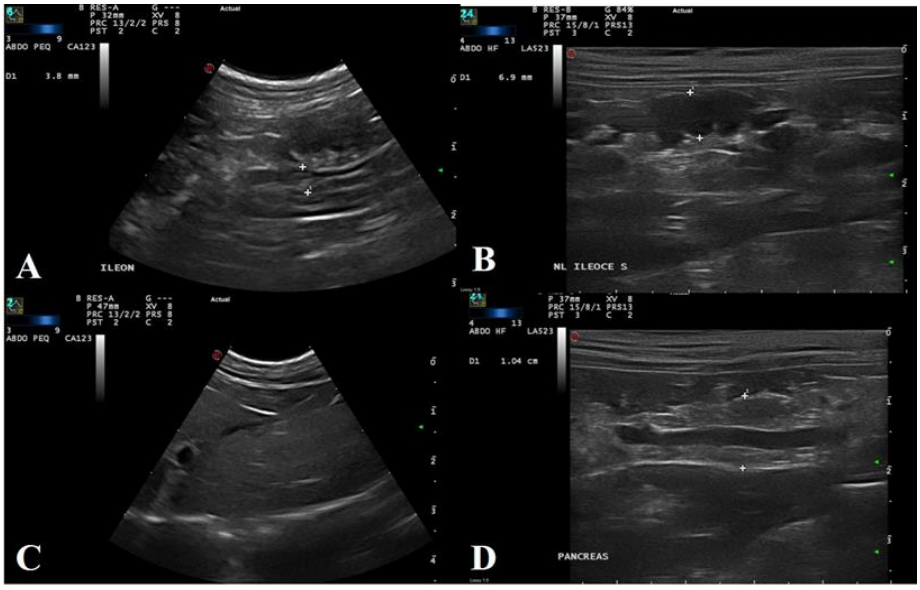

Figure 4. Abdominal ultrasound of a feline patient with triaditis. **(A)** Thickening of the muscularis wall of the ileum. **(B)** Mesenteric lymphadenopathy. **(C)** Prominent hepatic portal veins. **(D)** Mild pancreatitis.

Fraiche et al. (2016) reported a relatively sensitivity of the ultrasound for intestinal and pancreatic lesions (50-80%) but low for hepatic lesions (20-25%) in comparison with histological abnormalities in feline alimentary tract disorders. However, a good specificity was observed for the intestinal (57-100%) and liver lesions (71-80%) but not for the pancreatic ones (17-22%). Therefore, according to the results of this study biopsies of multiorgans are advice (Fraiche et al., 2016). Regarding intermediate grade AL (IHAL) and HGAL, the ultrasonographic features include transmural intestinal thickening with disruption of normal wall layering, reduced wall echogenicity, localised hypomotility and abdominal lymphadenomegaly (Grooters etal.,

1994). Moreover, transmural intestinal thickening in I/HGAL is usually symmetrical or concentric, in contrast to intestinal mast cell tumours and adenocarcinomas where intestinal wall thickening is often asymmetrical and extraintestinal involvement (Barrs and Beatty, 2012a). The ultrasonographic features of LGLL in cats seems to be similar to I/HGAL (Krick et al., 2008). A recent study described that that cats with round cell tumour, mainly lymphoma, tended to show hyperechoic perinodal fat but not statistically difference was observed in comparison to reactive lymph nodes (Davé et al., 2017).

3.5.3 Cytology

I/HGAL and LGLL may be diagnosed by ultrasound guided fine needle aspiration cytology from an intestinal mass and/or enlarged mesenteric lymph nodes (Barrs and Beatty, 2012a; Russel et al., 2012; Finotello et al., 2017) (Figure 5A). A recent study has proposed that H/IGAL is more common than LGAL when the cases diagnosed by cytology are included in addition to those diagnosed by histology in am Australian study population, because most of the published studies consider only histopathologic assessment (Russel et al., 2012). On the other hand, cytology obtained of LGAL from diffusely thickened wall is technically difficult and usually non-diagnostic as well as mesenteric lymph nodes because it is not easy distinguish well-differentiated neoplastic lymphocytes from benign lymphoid hyperplasia as occur in the

IBD (Lingard et al., 2009) (Figure 5B). Recently, a study evaluated the diagnostic value of cytology of endoscopic biopsies using the squash smear technique in cats described that the sensitivity of cytology to diagnose LGAL is low and it has no additional significance to histological biopsies (Mangelsdorf et al., 2015).

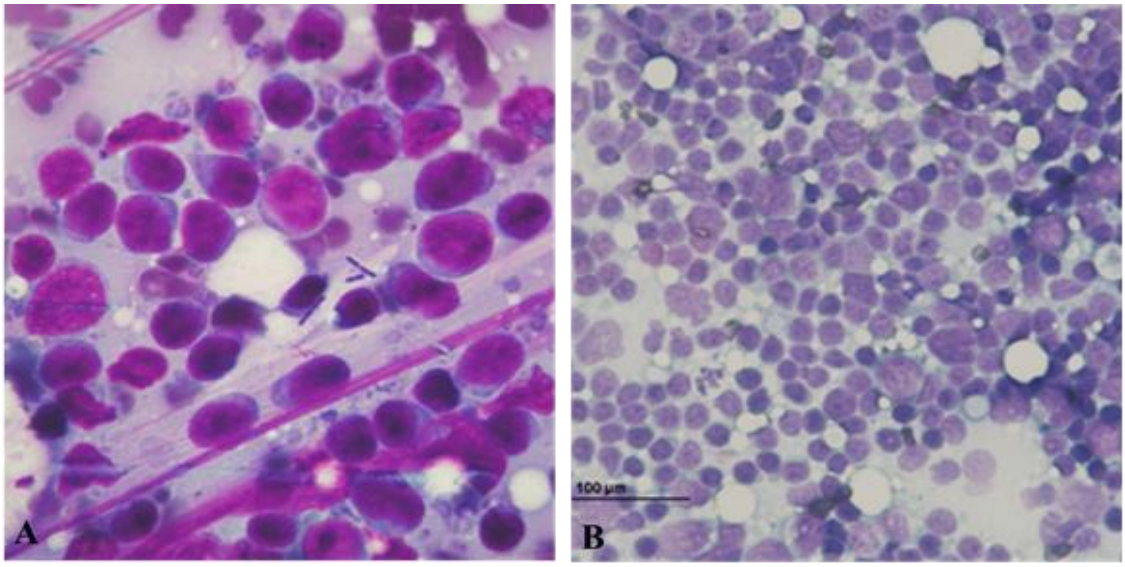

Figure 5. (A) Hight grade alimentary lymphoma diagnosed by cytology from an intestinal mass of a cat. **(B)** Cytology of lymphoid hyperplasia from mesenteric lymph node of a cat with IBD and lymphadenopathy.

3.5.4 Biopsy techniques

The decision about which organs should be biopsied is indicated by the clinical signs explained above to decide what segments should be biopsied such as stomach, small and/or large intestine. Currently, controversial issues exist regarding the ideal technique to obtain GI biopsies in cats for the histological diagnosis of IBD and LGAL.

The techniques described are laparotomy or laparoscopy and endoscopy (Figure 6A and B). In the Table 4, advantages and disadvantages of each technique are summarized.

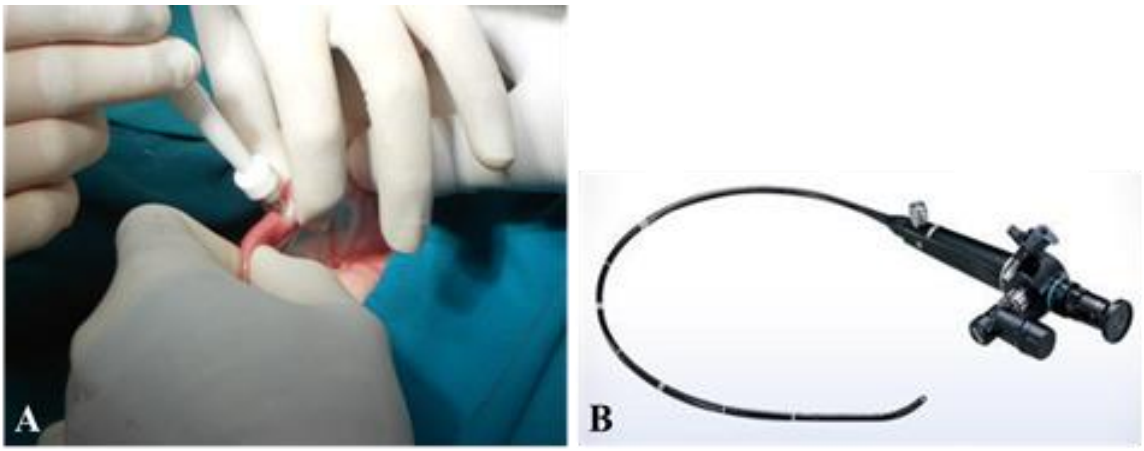

Figure 6. (A) Intestinal biopsy obtained by laparotomy in a cat. **(B)** Endoscope.

Endoscopy is a good technique to obtain tissue samples but it has some limitations. Endoscopic biopsy specimens are small and delicate compared to surgically obtained tissue samples. Other factors are related to inadequate operator experience, poor endoscopic biopsy techniques, processing of samples, and non-uniform histopathologic grading criteria, all of which negatively impact on the correct diagnosis (Willard et al., 2008; Day et al., 2008; Willard et al., 2010; Slovak et al., 2014). In one small series, histological evaluation of full-thickness biopsies (FTB) of the GI tract was found to be more sensitive than endoscopic biopsy for the diagnosis of LGAL (Evans et al., 2006). However, in that study, technical difficulties may have hampered the quality

of endoscopic biopsy specimens since some cats underwent only partial duodenal assessment or blind duodenal biopsy. The quality of endoscopic biopsy samples has a profound effect on their sensitivity for identifying certain lesions (Willard et al., 2008). The quality is classified as inadequate, marginal and adequate according to the structures observed in the histopathology mainly in biopsies obtained by endoscopy. Inadequate is defined as samples that have only superficial mucosa and epithelium, or deep mucosa, but not both; marginal is defined as samples that have epithelium and mucosa, but did not clearly have full-thickness mucosa; adequate is defined as samples that have full-thickness mucosa, whether or not it included muscularis mucosa (Willar et al., 2008) (Figure 7).

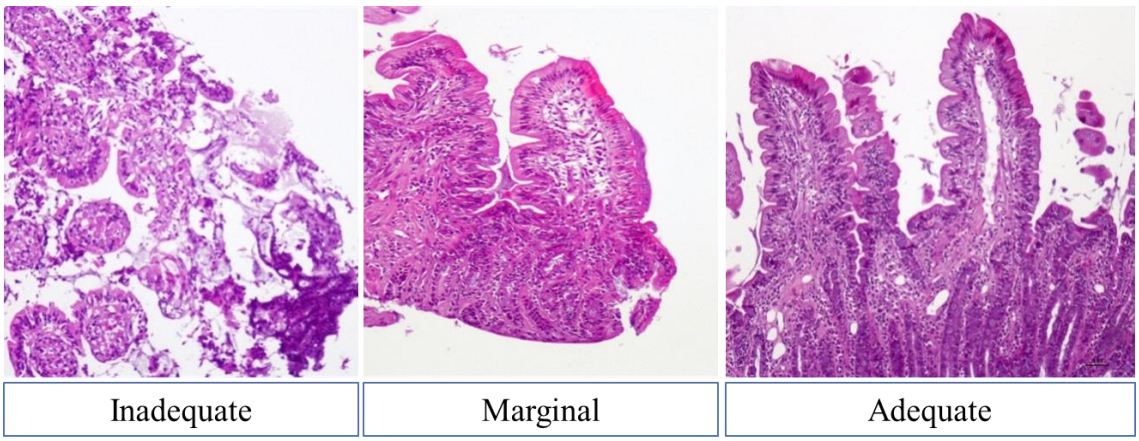

Figure 7. Quality of the endoscopy biopsies from the duodenum: inadequate, marginal and adequate.

Table 4. Comparison of full-thickness with partial-thickness intestinal biopsies for diagnosis of IBD and LGAL (modified from Barrs and Beatty, 2012).

Biopsy	**Full-thickness biopsy**		**Partial thickness biopsy**
Technique	**Laparatomy**	**Laparoscopy**	**Endoscopy**
Intestinal wall layers sampled	Mucosa, submucosa, muscularis, serosa		Mucosa, submucosa
Assessment of other organs	Visual inspection of serosal surface of GI tract and inspection/biopsy of other abdominal organs		Visual assessment of mucosal surface of gastrointestinal tract
Degree of invasiveness	Highest; longer hospital stays	Intermediate	Lowest; shorter hospital stays
Gastrointestinal regional accessed	All segments	All segments, but only jejunum biopsied routinely	Gastroduodenoscopy: stomach and duodenum. Colonoscopy: colon and ileum. Jejunum is not accessible
Requirements for timing of treatment	Delay (about 7 days) required because of risk of intestinal dehiscence during wound healing		No delay required
Operator skill	Advanced training not required	Advanced training required	Advanced training required
Pathologist skill and interpretation of biopsies	More likely to be oriented in the correct plane than endoscopic biopsies, aiding interpretation. Less subject to artefact		Variable quality, hamper interpretation. Greater level of pathologist expertise required for correct interpretation of poor quality endoscopic biopsies

It has been shown that if six marginal or adequate quality duodenal or gastric endoscopic biopsies are taken, as defined by the presence of at least one villus and subvillus lamina propria, correct histological diagnosis is very likely to be achieved (Willard et al., 2008). Number of biopsy samples recommended are summarized in Table 5 (Willard et al., 2008; Jergens et al., 2016). Furthermore, optimal histological processing, including biopsy orientation, positioning and staining, is also essential for correct interpretation (Willard et al., 2010). Day et al. (2008) attempted to standardize interpretation of IBD between pathologists that resulted in the design of a histology template for the WSAVA that defines numerous morphologic and inflammatory features in endoscopic biopsies that it is argued below. Despite all these, endoscopy abnormalities do correlate with FCEAI and histopathologic lesions at IBD diagnosis in cats when endoscopy is performed by an expert veterinarian (Figure 8) (Jergens et al. 2010). It is important to remind that ileum should be always biopsied when endoscopy is performed since lymphoma may be only placed there and misdiagnosis may occur (Scott et al., 2011). Furthermore, only proximal jejunal biopsies may be obtained by endoscopy thus if the main ultrasonography lesions are placed in the jejunum, surgery is recommended. The author of this review recommends reading a recent article about the diagnostic utility of endoscopy biopsy in dogs and cats with GI disease published by Jergens et al. (2016).

Table 5. Endoscopic guidelines for cats with chronic enteropathy assuming that samples are at least marginal for histopathological evaluation (Willard et al., 2008; Jergens et al., 2016).

Gastrointestinal organ	Minimum number of biopsies required	Comments
Stomach	6 adequate	Six mucosal samples generally diagnostic
Duodenum	6 adequate	Six mucosal samples generally diagnostic
Ileum	3-5 adequate	Exact number unknown; blind forceps biopsies are OK
Colon	9-12 adequate	Obtain 3-4 biopsies from each colonic region

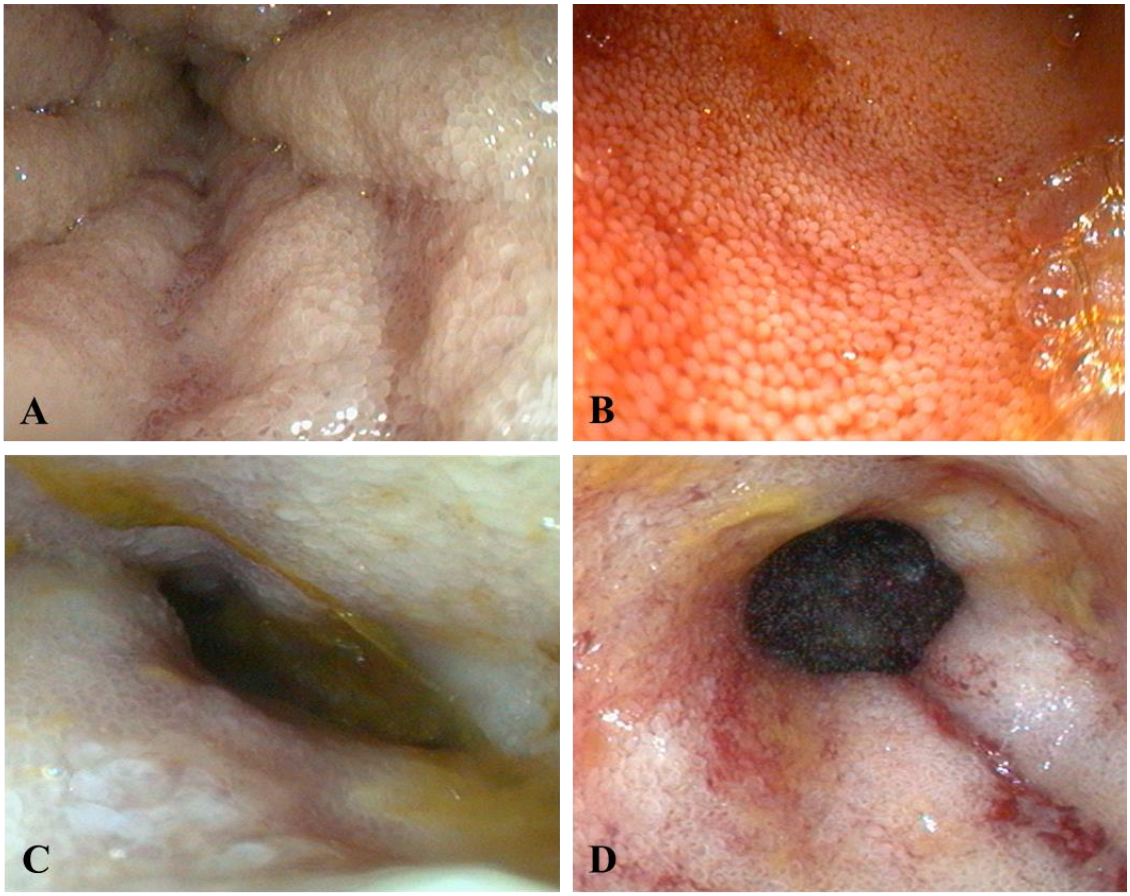

Figure 8. Endoscopy pictures from cats with IBD. **(A)** Increased granularity and few erythematous areas in the duodenum. **(B)** Increased granularity and bile content in the duodenum. **(C)** Increased granularity and mild erythema in the ileum. **(D)** Increased friability and granularity in the ileum. (Courtesy of Dr. Laura Fresno)

3.5.5 Histopathological diagnosis

The importance of differentiate between IBD and LGAL is mainly the prognosis. Cats with IBD have generally a good prognosis for control GI signs and a normal life span. Otherwise, LGAL has a guarded to poor prognosis. However, this differentiation is a diagnostic challenge to the clinicians and pathologists (Jergens, 2012). Furthermore, both diseases can coexist in the same cat (Scott et al., 2011; Kiupel et al., 2011). Firstly, it is important to know the histological structures that conform the small intestine (Figure 9).

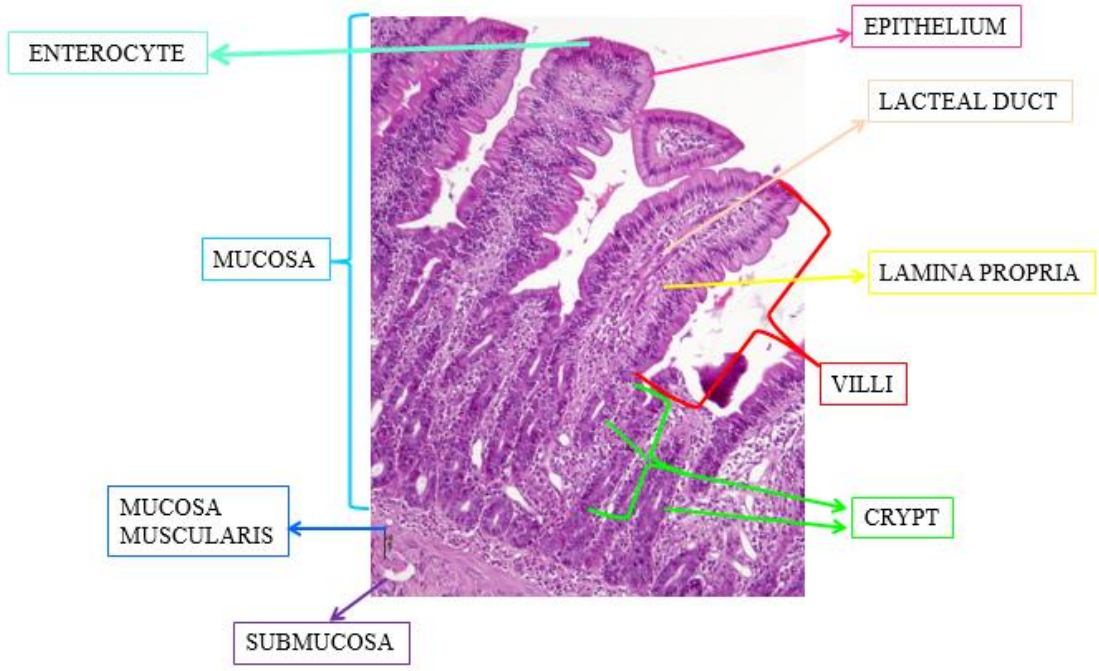

Figure 9. Histological structures that conform a normal (haematoxylin and eosin).

The microscopic findings in IBD consist of minimal to pronounced inflammatory cell infiltration of the gastric and/or intestinal mucosa accompanied by varying degrees of mucosal architectural disruption (Jergens, 2012). On the other hand, LGAL presents neoplastic infiltrates of small lymphocytes that are often indistinguishable from those cats with LPE (Barrs and Beatty, 2012b) (Figure 10A and B).

The WSAVA GI Standardization Group template proposed a standardization for histopathological analysis by a scoring template to evaluate the severity of inflammation, type of inflammation and morphological alterations of stomach, duodenum and colon using endoscopic biopsies (Day et al., 2008). Nowadays, templates

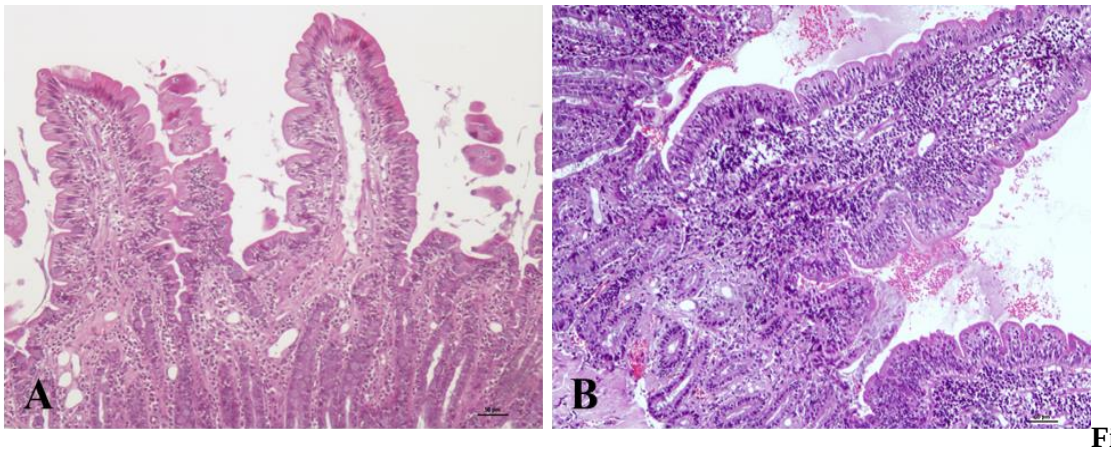

Figure 10. (A) Moderate lymphoplasmacytic enteritis diagnosed from duodenal endoscopy biopsy. **(B)** Low grade alimentary lymphoma from the jejunum of a cat.

for jejunal and ileal evaluation are needed (Procoli et al. 2013). At the same time, there are no templates for the FTB evaluation but the WSAVA scoring has been used in this type of biopsies and to evaluate ileum of dogs (Casamian-Sorrosal et al., 2010). These templates are showed by Figure 11. However, even with this standardized template there has been considerable disagreement between pathologists because it likely does not reflect the severity in dogs and cats. A new simplified pathologic model, using the WSAVA criteria that showed the most consistency in interpretation and including enumeration of goblet cells, has been recently proposed for dogs that shows a positive correlation with the clinical activity index and better agreement between pathologists (Jergens et al., 2014; Allenspach et al., 2017). Despite this, one study found correlation between the FCEAI and previous WSAVA score whose biopsies were evaluated by 1 certified pathologist in cats (Jergens et al., 2010). However, interobserver disagreement has been described by Willard et al. (2010) about histopathological diagnosis. This variation of the diagnosis was also present in cats when GI biopsies were evaluated by 3 certified pathologists of our institution using the WSAVA score thus disagreement between pathologists exists about this template in cats (Castro-López et al., 2013).

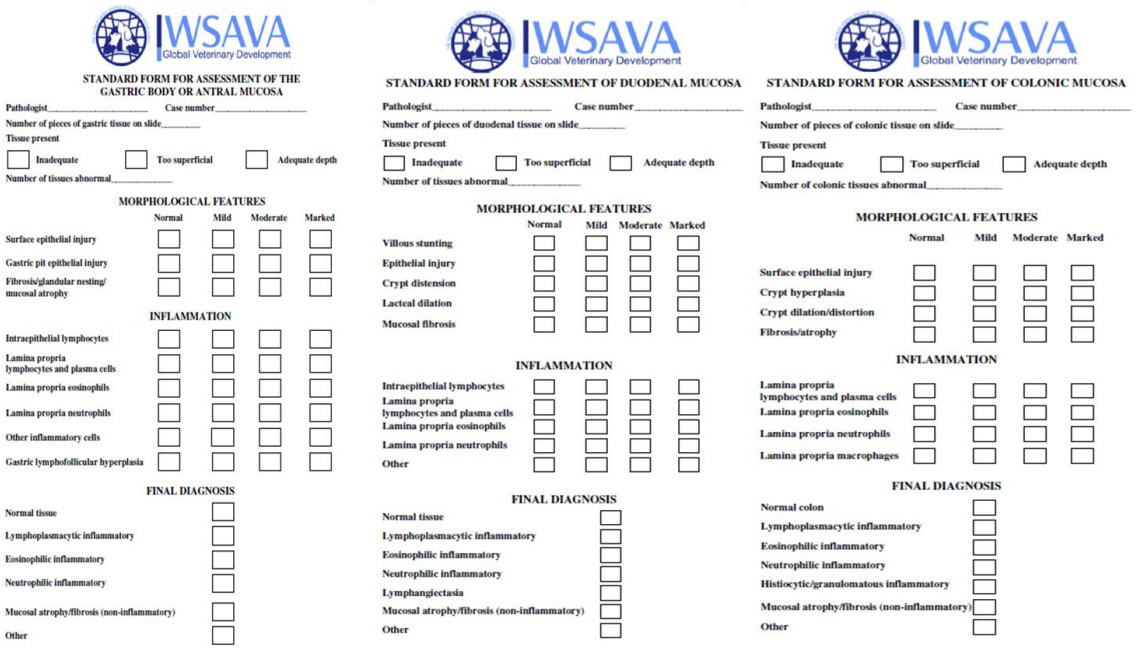

Figure 11. WSAVA template designed by the WSAVA Gastrointestinal Standardization Group for endoscopy biopsies of stomach, duodenum and colon (Day et al., 2008).

Histolopathologic lesions of IBD are subjectively classified based on the predominant cellular infiltrate within the lamina propria. The types of inflammation described in cats with IBD are: lymphoplasmacytic, eosinophilic, neutrophilic, and histiocytic. However, morphological alterations should be considered in IBD as well as LGAL since villous stunting, epithelial injury and crypt distension have been proposed by the modified WSAVA template to evaluate LGAL and to compare it with IBD (Maunder et al., 2012). These morphological abnormalities are showed in the Figure 12.

Lymphoplasmacytic infiltration is the most common inflammation found in lamina propria of feline IBD (Jergens et al., 1992; Dennis et al., 1993; Hart et al., 1994; Baez et al., 1999; Willard, 1999; Jergens et al., 2010). The second type more frequent is the eosinophilic enteritis, followed by the neutrophilic enteritis and finally granulomatous colitis (Tucker et al., 2014; Maunder et al., 2016; Van Kruiningen et al., 1979; Oliveira Leal et al., 2017).

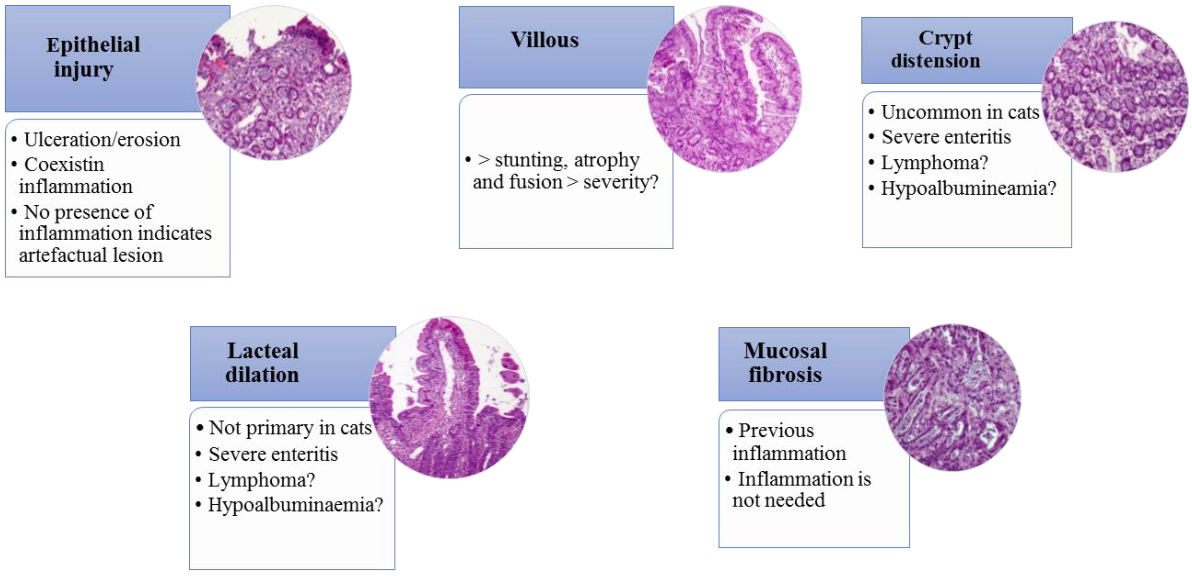

Figure 12. Morphological abnormalities according to the total and modified WSAVA and considerations (Day et al., 2008).

Histologic diagnosis of LPE is based on severity grading system of inflammatory infiltration (Janeckzo et al., 2008; Jergens, 2012). The WSAVA score system attempts to standardize interpretation of GI inflammation and classified according to severity of

cellular infiltration (Day et al., 2008). However, cats without GI clinical signs can present with inflammatory infiltration (Janeckzo et al., 2008; Fragkou et al., 2016). Classification of infiltration and differential diagnosis are showed in Figure 13.

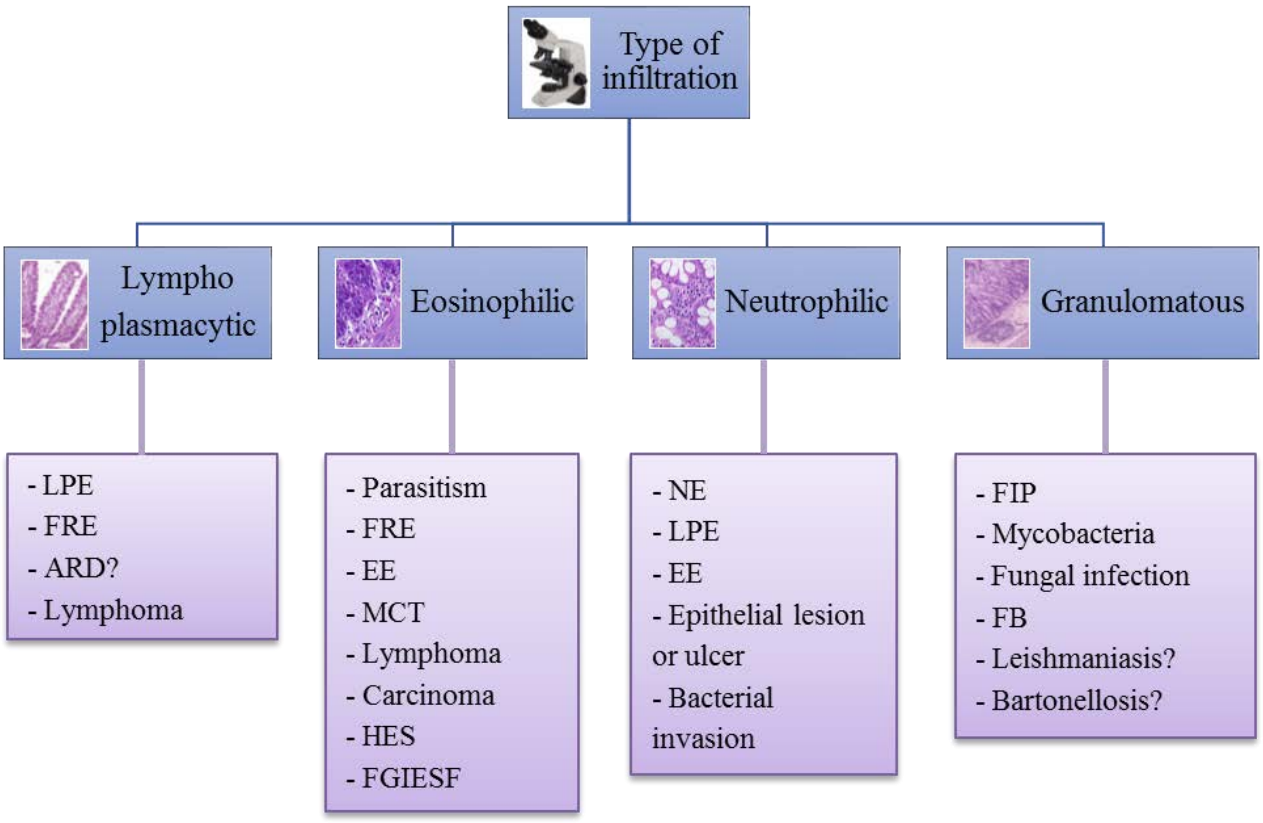

Figure 13. Differential diagnosis according to the type of infiltration. LPE: lymphoplasmacytic enteritis; FRE: food responsive diarrhoea; ARD: antibiotic responsive diarrhoea; EE: eosinophilic enteritis, MCT: mast cell tumour; HES: hypereosinophilic syndrome; FGIESF: feline gastrointestinal eosinophilic stromal fibroplasia; NE: neutrophilic enteritis; FB: foreign body.

Otherwise, a severe infiltration should be evaluated with caution and it is a challenge for the pathologist because it must be differentiated of LGAL.

Eosinophilic enteritis is characterized by a moderate and marked eosinophils infiltration in the lamina propria along or not a lower proportion of lymphoplasmacytic or lymphocytic inflammation (Tucker et al., 2014). This diagnosis was performed by the observation of eosinophils in the submucosa and the presence of infiltrates in the mesenteric lymph nodes (Wilcock, 1992). However, the WSAVA score easily and objectively classified the eosinophilic infiltration according to the severity of infiltration (Day et al., 2008). The most common clinical signs are vomiting, anorexia, and weight loss, and thickened intestinal loop on palpation (Tucker et al., 2014). Some cats with eosinophilic enteritis may show peripheral eosinophilia, when this is present along with muscularis wall thickening and lymphadenopathy in the ultrasound is suggestive to eosinophilic enteritis (Tucker et al., 2014). This enteritis may occur alone or in association with hypereosinophilic syndrome (Jergens, 2012). This syndrome produces eosinophilic infiltration in the intestine, liver, spleen and/or bone marrow.

Neutrophils are usually only a minor component of the inflammation observed in LPE or erosive lesion of the epithelium. Colitis affects frequently to young cats and enteritis to adults or older cats (Leib et al., 1986; Maunder et al., 2016). Clinical signs are vomiting, diarrhoea, haematoquezia and tenesmus (Jergens, 2012). This diagnosis

was made by the observation of dense infiltrates of neutrophils accompanied by lesser numbers of lymphocytes and plasma cells (Jergens, 2012). However, the current WSAVA score classified the neutrophilic infiltration according to the severity score to diagnose this enteropathy (Day et al., 2008). The cause is unknown but recently it has been associated with *Campylobacter coli* which attracts neutrophils. Further studies are needed to determine if only antibiotics are needed for the treatment.

Granulomatous colitis has been reported only in 2 cats, the most recent case was associated with intracellular *Escherichia coli* detected by fluorescence in situ hybridization (Van Kruiningen et al., 1979; Oliveira Leal et al., 2016). Further studies are needed to determine if only antibiotics are needed for the treatment.

AL is characterised by infiltration of the GI tract with neoplastic lymphocytes, with or without mesenteric lymph node involvement (Barrs and Beatty, 2012a). The histological classification systems most frequently applied to feline lymphoma are the National Cancer Institute Working Formulation (NCIWF) classifies lymphoma according to its natural rate of progression, and recognizing three histological grades (low, intermediate and high) based mainly in the size of the cell and number of mitoses (Valli et al., 2011). The other classification is the Revised European-American Lymphoma/World Health Organisation (REAL/WHO) scheme which includes specific

disease entities based on immunophenotyped and morphological features (Harris et al., 1994). These all schemes are complementary.

According to NCIWF, LGAL is presented when the number of mitoses is between 0-5 at high-power field and small nuclear size (<1.5X the size of a red blood cell) (Valli et al., 2011). Synonymous are well-differentiated, lymphocytic, and small cell lymphoma. Using the REAL/WHO classification is categorized in epitheliotropic T cell lymphoma, epitheliotropic small T cell lymphoma, intestinal T cell lymphoma and enteropathy-associated T cell lymphoma (Barrs and Beatty, 2012). The distribution of GI involvement in LPE is similar to LGAL except that gastric involvement is more common in LPE and LGAL is placed commonly in the jejunum or ileum (Evans et al., 2006; Lingard et al., 2009; Briscoe et al., 2011).

Differentiating IBD from LGAL in cats is a diagnostic challenge to both veterinary clinicians and pathologists, mainly in endoscopic biopsies. This differentiation is based on the histologic evaluation of intestinal biopsies, but it can be difficult to distinct between both diseases by histomorphology alone (Figures 14A and B) (Carreras et al., 2003; Willard et al., 2002; Moore et al., 2005; Kiupel et al., 2010; Briscoe et al., 2011).

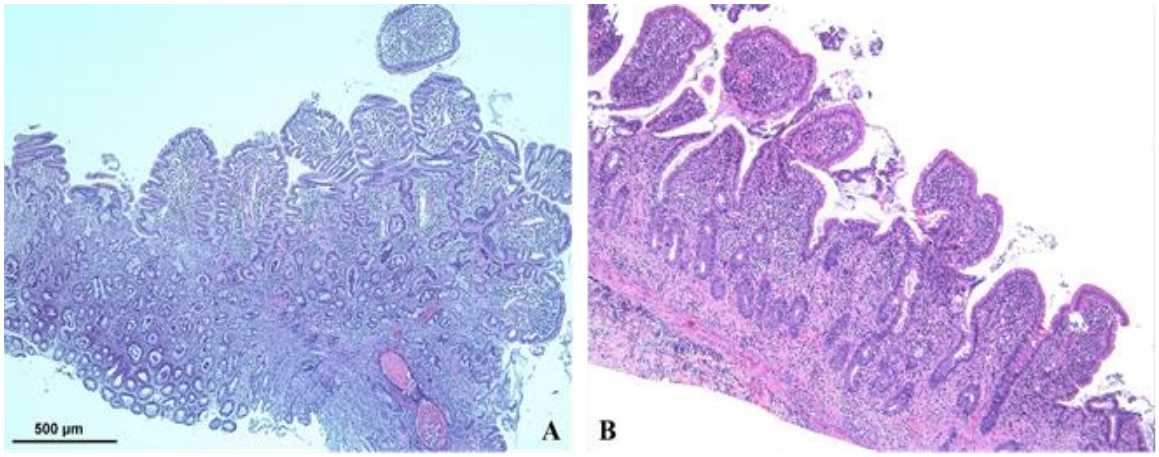

Figure 14. Endoscopy samples that morphological features and infiltration were similar between both biopsies, immunohistochemistry was used to differentiate between IBD and lymphoma. **(A)** Severe lymphoplasmacytic enteritis from the duodenum of a cat. (B) Low grade alimentary lymphoma from the duodenum of a cat.

T cell LGAL and IBD are both characterized by marked infiltrates of small lymphocytes that cannot be distinguished by histology alone (Carreras et al., 2003; Moore et al., 2005; Briscoe et al., 2011). In addition, lymphoma and inflammatory infiltrates frequently coexist (Moore et al., 2005; Scott et al., 2011). In a histopathological review of LPE and LGAL, there was disparity in diagnosis between two pathologists of moderate to marked LPE, requiring assessment by a third pathologist to reach a consensus diagnosis (Briscoe et al., 2011). However, in contrast to IBD, neoplastic T cells often infiltrate beyond the mucosa into the submucosa, tunica

muscularis, and serosa, destroying normal tissue architecture but when intestinal specimens are obtained by endoscopy, histologic evaluation is often limited to the mucosa (Kiupel et al., 2010). Histological criteria distinguishing LGAL from LPE include, in the former, the relative absence of mixed lymphoid and granulocytic cells and their replacement with monomorphous sheets of neoplastic lymphoid cells involving the lamina propria. In early disease, neoplastic cells form lamina propria patches, which are discrete regions of lymphocytic infiltration within some villi but not others (Valli et al., 2000; Ritcher, 2003; Brown, 2007; Moore et al., 2012). As disease progresses, lamina propria bands of lymphocytes spanning the crypt-villous junction are seen, followed by villous laminal propria obliteration by dense, monomorphic, lymphocyte infiltrates. In the most severe lesions villous and crypt laminal propria obliteration occurs due to complete lymphocytic infiltration and formation of a band of lymphocytes beneath the crypt epithelium but above the muscularis mucosae (Moore et al., 2012). Epitheliotropism is characterized by increased numbers of intraepithelial lymphocytes (IELs) and is a feature of both diseases (Hart et al., 1994; Waly et al., 2005; Day et al., 2008; Washabau et al., 2010; Briscoe et al., 2011; Kiupel et al., 2011). However, patterns of epitheliotropism, including the formation of nests (≥ 5 clustered IELs) or plaques (≥ 5 adjacent epithelial cells overrun by IELs) in the villous or crypt epithelium, are highly specific for LGAL (Fondacaro et al., 1999; Carreras et al., 2003; Richter, 2003; Briscoe et al., 2011; Kiupel et al, 2011; Moore et al., 2012). Other

histological features of LGAL that help to differentiate this disease from LPE include extension of the lymphocytic infiltrate into layers deep to the mucosa more severe disruption to villous and crypt architecture, intravascular lymphocytic infiltrates, high mitotic index and the presence of neoplastic cellular infiltrates in mesenteric lymph nodes (Valli et al., 2000; Ben-Ezra, 2001; Briscoe et al., 2011; Kiupel et al., 2011). Kiupel et al. (2011) reported that intraepithelial surface plaques, intravascular infiltrate, serosal infiltration, crypt intraepithelial nests and plaques, metastasis are 100% specific of LGAL.

3.5.6 Immunohistochemistry

Immunophenotyping has become an important diagnostic tool in differentiation between IBD and LGAL when histologic changes are ambiguous (Waly et al., 2005) (Figure 15A and B). Routine immunohistochemistry (IHC) used are CD3 for detection of T cells and CD79a for B cells, CD20 also can be used for detection of B cells. A monomorphic lymphocytic population supports a diagnosis of lymphoma, while a mixed lymphocytic population supports a diagnosis of inflammation (Barrs et al., 2012b) (Figure 15A, B, C and D).

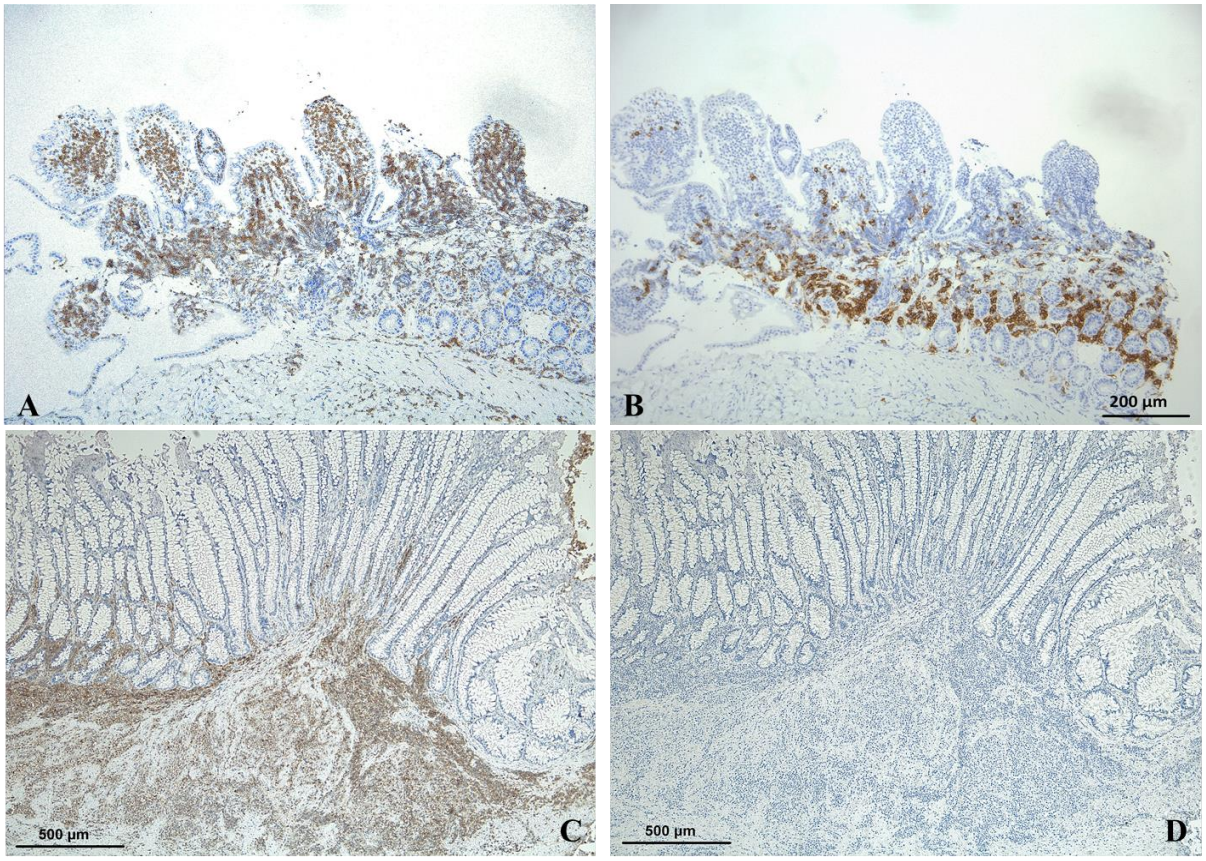

Figure 15. Immunohistochemistry of **(A)** CD3 positive T cells are normally distributed in the epithelium and villi from severe lymphoplasmacytic enteritis. **(B)** CD20 positive B cells are normally distributed in the mucosa-associated lymphoid tissue from the same severe lymphoplasmacytic enteritis. **(C)** CD3 positive T cells distributed abnormally in the all ileal mucosa from low grade alimentary lymphoma. **(D)** CD20 negative B cells in all ileal mucosa from the same low grade alimentary lymphoma.

Waly et al. (2005) described that 5 of out 32 cats diagnosed with lymphoma based on haematoxylin and eosin (H&E) stained sections, immunohistochemical stains revealed that infiltrate was composed of a mixed population of small B and T lymphocytes and plasma cells thus these cases were reclassified as LPE. Furthermore,

Kiupel et al. (2011) also described that 5 of 19 cases classified as LPE based on H&E stained were reclassified as T cell LGAL when IHC was performed. Importantly, LGAL cannot be diagnosed based on T cell phenotype alone, since expansion of T cell populations in intestinal mucosal-associated lymphoid tissue can occur in inflammatory intestinal disease in cats (Moore et al., 2005; Evans et al., 2006; Kiupel et al., 2011). Therefore, PCR is needed in these cases.

An additional feature of CD3 stained intestinal sections is that IELs can be more clearly visualised than in H&E stained sections. Other features should be considered, primary small intestinal T cell LGAL is most common over 90% of the cases whereas I/HGAL show variable T or B cell origin (Jackson et al., 1996; Gabor et al., 1998; Vail et al.,1998; Zwahlen et al., 1998; Patterson-Kane et al., 2004; Waly et al., 2005; Kiupel et al., 2011; Moore et al., 2012). In addition, a strong association between immunophenotype and location within the GI tract has been identified, B cell lymphoma is located mainly in the stomach, cecum and large intestine while T cell lymphoma is most commonly found in the small intestine (Moore, 2006; Pohlam et al., 2009; Moore et al., 2012).

It has been reported that cats with IBD and intestinal lymphoma presented overexpression of Bcl-2 apoptopic marker but no difference was observed between both

diseases though cats with intestinal lymphoma tended to show higher percentage but overlap existed between IBD and LGAL (Swanson et al., 2012).

3.5.7 PCR for antigen receptor rearrangement (PARR)

Determination of clonality of T cell populations in lymphocytic intestinal infiltrates is a useful diagnostic tool when the distinction between LGAL and LPE remains ambiguous after histological evaluation and immunophenotyping. The clonality test performed is the PARR. Infiltrates of T lymphocytes in intestinal sections can be determined by assessment of T cell receptor gamma (TCRG) V-J junctional diversity. Similarly, B cell clonality can be determined by PCR of immunoglobulin heavy chain (IgH) variable region. During development in the thymus, T cells rearrange their antigen receptor genes TCRA, TCRB, TCRG and TCRD to form two lineages, αβ and δγ T cells. Most αβ T cells rearrange TCRG before rearrangement of TCRA and TCRB. The TCRD gene is deleted from the genome during rearrangement of the TCRA gene. Thus, TCRG gene rearrangements occur in both αβ and δγ T lymphocytes. During this process, the V-domains are somatically rearranged in a process called V-J recombination, where the V-region is randomly and imprecisely joined to the J-region. Random nucleotides are added at the joining sites, further enhancing diversity and length polymorphism. This length polymorphism can be visualised by conventional PARR of the resultant hypervariable region of the V-domain known as the

complementarity determining region 3 (CDR3), using primers directed against relatively conserved framework regions. Amplified products are analysed using heteroduplex gel electrophoresis and clonality is determined by the number and size of the bands in duplicate samples run side by side. Clonal lymphocyte populations produce one or two sharp bands that are consistent in duplicate samples; oligoclonal populations produce three bands; while polyclonal populations produce a broad band, smear or ladder of bands. Pseudoclonal populations contain one or two bands that are of different sizes or are non-reproducible in duplicate analyses. Neoplastic populations of T lymphocytes are clonal or oligoclonal, while inflammatory populations are polyclonal (Moore et al., 2005).

In three studies, determination of lymphocyte clonality by PARR was 78 to 90% sensitive in the detection of intestinal T cell lymphoma, where clonal or oligoclonal T cell populations were considered to be neoplastic (Moore et al., 2005; Weiss et al., 2011b; Moore et al., 2012). In one of these studies, 22 of out 28 cats were found to have clonal rearrangements of the TCRG gene, while three had oligoclonal rearrangements. In comparison, polyclonal rearrangements were detected in 3 of 3 cats with normal intestinal histology and in 9 of 9 cats with LPE (Moore et al., 2005). Performing concurrent T and B cell clonality analysis can increase the sensitivity of detection of T cell lymphomas due to cross-lineage gene rearrangements (Kiupel et al., 2011; Sato et al., 2011). In this study, when IHC was added to H&E stained, 5 cats diagnosed firstly

as IBD changed to LGAL, but when PARR was also added the number or LGAL increased to 10 (Kiupel et al., 2011). Otherwise, 1 case was reclassified as IBD with H&E plus IHC, when the original diagnosis was LGAL, but when PARR was also used 3 cases changed from LGAL to IBD diagnosis (Kiupel et al., 2011).

As clonality is not always specific for malignancy, this technique cannot be used as a stand-alone diagnostic test for LGAL. A diagnostic approach that combines the assessment of histological features, immunophenotype and clonality analysis is optimal to distinguish LGAL from LPE, especially when endoscopy biopsies are submitted (Kiupel et al., 2011). For this reason, Kiupel et al. (2011) proposed an algorithm for the diagnosis that is showed in the Figure 16. Furthermore, sensitivity and specificity of H&E plus IHC is 78% and 99%, respectively, but when PARR is added to the diagnosis, sensitivity increases to 82% (Kiupel et al., 2011). Lymphocyte lineage should be based on immunophenotypic assessment rather than clonality determination by PARR if the results of these two techniques are divergent (Moore et al., 2005). This is a key point, because PARR does not determine lymphocyte phenotype since as neoplastic lymphocytes may show clonal rearrangement of either or both the T or B cell antigen receptor genes, regardless of phenotype (Andrews et al., 2016). Moreover, a recent study described that 8.7% of the LGAL showed cross lineage rearrangement (Andrews et al., 2016).

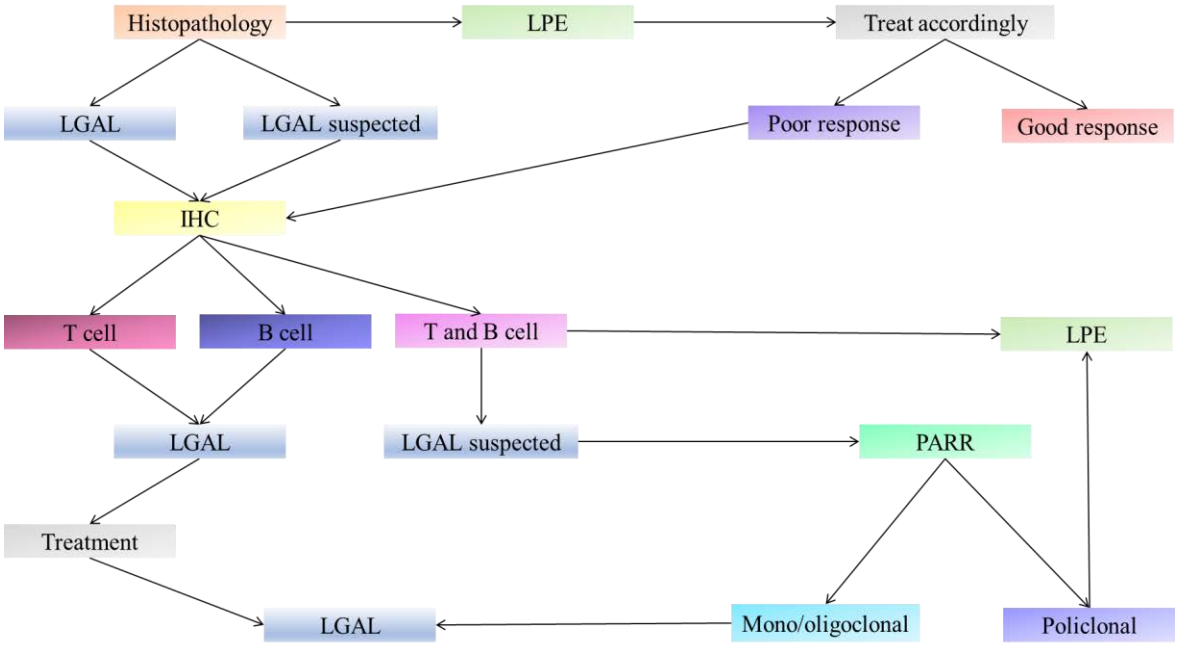

Figure 16. Diagnostic algorithm to differentiate feline intestinal lymphoma from inflammatory bowel disease in small intestinal surgical biopsy specimens (Modified from Kiupel et al., 2011). LPE: lymphoplasmacytic enteritis; LGAL: low grade alimentary lymphoma; IHC: immunohistochemistry; PARR: PCR for antigen receptor rearrangement.

3.6 P-glycoprotein and Cyclooxigenase-2: their implication in the intestine and lymphoma

Multidrug resistance 1 (*MDR-1*) gene and its product, permeability glycoprotein (P-gp), might be determinant in susceptibility to IBD, disease behaviour and response to therapy in humans (Ho et al., 2003). P-gp is an adenosine triphosphate-dependent membrane-bound efflux transporter pump, expressed in the intestinal epithelium and tumours leading to move different substrates and drugs from the inner to the outer leaflet of the cell membrane inducing resistance to anticancer agents (Fojo et al., 1987; Thiebaut et al., 1987; Fromm, 2002). Additionally, P-gp of the intestinal epithelium may protect against xenobiotics and bacterial products through the same mechanism (Fromm, 2002). Therefore, P-gp and *MDR-1* gene may be implicated in the pathogenesis of IBD (Fromm, 2002).

Epithelial P-gp expression is normal in small and large intestine of cats and humans, but in dogs is only expressed in colon (Van der Heyden et al., 2009; Cordon-Cardo et al., 1990; Van der Heyden et al., 2011b). Loss of the epithelial P-gp expression in lymphoplasmacytic colitis and expression in small intestine IBD described in dogs may be a cause or consequence (Van der Heyden et al., 2011b). As cause, mutant mice

lacking P-gp expression (*mrd1*a-/-) spontaneously develop colitis due to a defect in barrier function may increase accumulation of bacterial breakdown products within epithelial cells and/or immunological dysregulation of cytokines and chemokines in the intestine (Wilk et al., 2005). Otherwise as consequence, all morphological and inflammatory alterations destroy the intestinal epithelium or may reduce the P-gp production and expression like occur in rats (Buyse et al., 2005). Finally, no correlation has been observed between WSAVA score and P-gp expression in the epithelium (Van der Heyden et al., 2011b).

P-gp expression in the lamina propria is absent in healthy humans, cats and dogs (Van der Heyden et al., 2009; Cordon-Cardo et al., 1990; Allenspach et al., 2006). However, dogs with IBD can present P-gp overexpression in the inflammatory cells that infiltrate the lamina propria, and tumour cells of the canine and feline lymphoma (Brenn et al., 2008; Allenspach et al., 2006; Bergman et al., 1996; Ginn et al., 1996). Dogs with severe IBD that needed cyclosporine in the treatment showed higher P-gp expression in the lamina propria compared to the dogs that responded only to steroids (Allenspach et al., 2006). Therefore, it has been proposed that P-gp expression in the lamina propria is implicated in the resistance to treatment.

A study evaluated the P-gp expression in feline lymphoma as prognosis tool, but no correlation was found with prediction of remission and survival time and no all

samples were positive (Brenn et al., 2008). Pretreatment P-gp expression was found to be prognostic in older studies of canine lymphoma (Bergman et al., 1996; Lee et al., 1996). However, more recent studies were not able to confirm this in dogs (Dhaliwal et al., 2013; Gramer et al., 2013; Zandvliet et al., 2015). In human beings have been suggested a role for P-gp in mediating drug resistance in a subset of patients with refractory lymphoma but this has not been confirmed (Sandor et al., 1997).

Cyclooxygenase 2 (COX-2) is an inducible inflammatory regulator isoform by cellular activation, proinflammatory cytokines, growth factors, tumour promoters and prostaglandin mediator (Vane et al., 1998; Williams et al., 1999; Yu et al., 2007; Ghosh et al., 2010). Prostaglandin E2, a COX-2 metabolite, has many biological roles including mediating pain, modulation of cytokine production, induction of regulators of angiogenesis, production of proinflammatory mediators and promotes tumourigenesis (Funk, 2001; Charlier et al., 2003). Furthermore, overexpression of COX-2 may be a consequence of inflammation leading to increased levels of Bcl-2 and resistance to apoptosis of the cells, thus enhancing the risk of cancer (Tsujii et al., 1998; Sakamoto et al., 2005). There is only one available study in cats that included 6 cases of intestinal lymphoma and described negative COX-2 immunoexpression (Beam et al., 2003).

In healthy cats, only one study has described COX-2 immunoexpression in basal granulated cells of the epithelium of the GI tract using a polyclonal antiprostaglandin H

synthetase-2 (COX-2) human C terminus antibody (Satoh et al., 2013). Physiologic higher COX-2 expression has been described in feline duodenum after feeding (Satoh et al., 2013). On the other hand, it has been demonstrated that only 50 to 80% of healthy humans presents COX-2 expression in colon and stomach (Jackson et al., 2000; Paiotti et al., 2007; Romero et al.,2008; Dai, 2015).

Higher epithelial COX-2 immunolabelling has been reported in human beings with gastritis induced by *Helicobacter pylori*, ulcerative colitis or Crohn's disease compared to normal epithelium, likely secondary to GI epithelial ulceration (Singer et al., 1998; Jackson et al, 2000). Furthermore, increased mucosal levels of prostaglandin E2 in humans and interleukin-1β in dogs with IBD and food responsive diarrhoea have been linked to an increased COX-2 immunoexpression or upregulation (Singer et al., 1998; Dumusc et al., 2014). Based on these studies, it has been suggested that cytokines and prostaglandins induced by an inflammatory response increase COX-2 in the intestinal mucosa as a protective mechanism (Singer et al., 1998; Dumusc et al., 2014). In humans with IBD, macrophages and polymorphs are stained by COX-2 at the lamina propria (Singer et al., 1998; Roberts et al., 2001Paiotti et al., 2007; Romero et al., 2008; Dai et al., 2015). However, those inflammatory cells are uncommon in veterinary IBD.

Association between COX-2 upregulation and development of lymphoma, as occurs in some tumours, remains unknown but COX-2 overexpression is associated

with cell proliferation and angiogenesis (Joo et al., 2002; Joo et al., 2003; Ohsawa et al., 2006; Mohammed et al., 2004). A recent report stated that 15% of canine lymphoma presented COX-2 overexpression (Asproni et al., 2014). However, other studies in canine lymphoma did not find COX-2 immunoreactivity like normal lymph node (Mohammed et al., 2004; Rodrigues et al., 2011). In contrast, studies in humans revealed that most of non-Hodgkin's lymphoma (>50% of cases) had COX-2 expression by tumour cells (Hazar et al., 2006; Paydas et al., 2007; Ma et al., 2012). In addition, COX-2 upregulation in lymphomas has been associated with the aggressiveness, relapsed, worst response to therapy and less overall survival (Hazar et al., 2006; Paydas et al., 2007; Sugita et al., 2007; Ma et al., 2012). No correlations have been observed between clinical activity index, histological alterations with COX-2 expression in canine IBD, and human lymphoma and IBD (Hazar et al., 2006; Paiotti et al., 2007; Paydas et al., 2007; Ma et al., 2012; Dumusc et al., 2014).

4. STUDIES

4.1 STUDY I

P-glycoprotein immunoexpression in intestinal epithelium and lamina propria of cats with inflammatory bowel disease and low grade alimentary lymphoma

J. Castro-López, A. Ramis, M. Planellas, M. Teles and J. Pastor (Submitted)

ABSTRACT

Objective: P-glycoprotein (P-gp) has been associated with the development of inflammatory bowel disease (IBD) and generates resistance to the treatment in lymphoma. The aims of this study were to investigate the epithelial and lamina propria (LP) expression of P-gp in cats with IBD and low grade alimentary lymphoma (LGAL), and to correlate this expression with clinical signs and histopathological scoring.

Method: P-gp expression was evaluated by immunohistochemistry (IHC) in 9 cats with IBD (lymphoplasmacytic enteritis) and 10 with LGAL in the epithelium and lamina propria (LP). FCEAI, modified and total WSAVA were calculated.

Results: Fifty-six percent of the cats with IBD showed a continuous or normal P-gp expression in the epithelium whereas 80% of the cats with LGAL showed a discontinuous or abnormal one (χ^2= 11.52; p= 0.003). When contingency table was applied, the LGAL group showed frequently a discontinuous epithelial expression compared to other groups (p< 0.003). Lower number of the cats with IBD presented with marked P-gp expression (44%) in the LP compared to cats with LGAL (60%), but 22% of cats with IBD showed moderate P-gp immunolabelling in the LP and 10% of cats with LGAL a mild one. When contingency table was applied, the IBD group was frequently distributed showing a moderate expression in the LP whereas the LGAL group tended to show frequently a marked one (p= 0.03; p= 0.05, respectively).

Correlation was observed between P-gp expression in the LP and FCEAI, total and modified WSAVA scores (Spearman's $\rho > 0.594$; $p < 0.001$) but not between epithelial P-gp expression and any variables.

Conclusions: Most of cats with LGAL presented with a higher FCEAI and modified WSAVA as well as discontinuous P-gp immunoexpression in the intestinal epithelium and marked immunostaining in the LP in comparison with cats with IBD. Nonetheless, 66% of cats with IBD showed P-gp immunolabelling in the LP and correlation between expression and histopathological alterations was observed. Therefore, P-gp expression might be implicated in the pathogenesis, severity, resistance to treatment and prognosis of both diseases and further studies are needed to elucidate these conclusions.

INTRODUCTION

Inflammatory bowel disease (IBD) and low grade alimentary lymphoma (LGAL) are common causes of chronic enteropathies (CEs) in cats (Guilford et al., 2001; Fondacaro et al., 1999; Jergens et al., 2010; Barrs and Beatty, 2012a; Jergens, 2012; Norsworthy et al., 2013). IBD is a chronic immune-mediated inflammation with unknown aetiology likely to be multifactorial (Guilford et al., 2001; Fondacaro et al., 1999; Allenspach et al., 2007; Jergens et al., 2010; Jergens, 2012). Nowadays, AL is the most common lymphoma in cats in the post-feline leukemia virus era and the cause remains also unknown (Barrs and Beatty, 2012a; Vail et al., 1998; Bertone et al., 2002; Louwerens et al., 2005; Milner et al., 2005; Stutzer et al., 2011). Furthermore, evolution from chronic intestinal inflammation to AL has been proposed as a risk factor but a definitive proof is still lacking (Louwerens et al., 2005; Mahony et al., 1995).

Clinical differentiation between IBD and low grade alimentary lymphoma (LGAL) may be a challenge. Therefore, histopathological diagnosis is needed though overlapping may occur, thus additional techniques such as immunohistochemistry can be helpful (Barrs and Beatty, 2012b; Moore et al., 2005; Briscoe et al., 2011; Kiupel et al., 2011; Willard et al., 2002). Definitive diagnosis is imperative for adequate treatment and prognosis (Jergens, 2012; Barrs and Beatty, 2012b; Kiupel et al., 2011; Willard et al., 2002). In addition, resistance to the treatment may be observed in cats with severe

IBD and LGAL thereby a more aggressive treatment is needed (Jergens, 2012; Barrs and Beatty, 2012b).

Multidrug resistance 1 (*MDR-1*) gene and its product, permeability glycoprotein (P-gp), might be determinant in susceptibility to IBD, disease behaviour and response to therapy in humans (Ho et al., 2003). P-gp is an adenosine triphosphate-dependent membrane-bound efflux transporter pump, expressed in the intestinal epithelium and tumours leading to move different substrates and drugs from the inner to the outer leaflet of the cell membrane inducing resistance to anticancer agents (Fojo et al., 1987; Thiebaut et al., 1987; Fromm, 2002). Additionally, P-gp of the intestinal epithelium may protect against xenobiotics and bacterial products through the same mechanism (Fromm, 2002). Therefore, P-gp and Mdr-1 gene may be implicated in the pathogenesis of IBD (Fromm, 2002). According to authors' knowledge, there is not report about P-gp expression in feline IBD. A previous study reported about P-gp expression in feline lymphoma, but only one case of LGAL was included (Brenn et al., 2008).

The first aim of this study was to investigate P-gp expression in the epithelium and lamina propria (LP) of cats with IBD and AL. The second objective was to correlate the P-gp expression with clinical signs and histopathological scoring.

MATERIAL AND METHODS

Study population

Control animals were composed by: 1) 3 duodenal biopsies obtained by endoscopy immediately before ovariohysterectomy from 3 healthy female control cats (HCC) that they were indoor owned cats (mean age= 2.6-year-old) prolonging the anaesthetic procedure about 25 minutes; 2) Full thickness biopsies (FTB) of duodenum, jejunum and ileum from 5 owned sick cats (SC) (mean age= 7.8) euthanized or died for natural causes not related or that not affect to gastrointestinal tract were obtained within 1 hour post-mortem. These cats did not receive any drugs that induce the P-gp expression at least 6 months before sampling. Approval consent was signed and accepted by the owners of the HCC and SC, and procedures were approved by the Ethical Committee from the Universitat Autònoma de Barcelona (UAB; CEAAH 2354).

Cats with IBD or LGAL presented between June 2007 and January 2013 to the Veterinary Teaching Hospital of the UAB were included in the study. The inclusion criteria were the presence of chronic gastrointestinal signs (>3 weeks duration), complete medical history and no administration during the last year of glucocorticosteroids or drugs that induces P-gp expression (Mealey and Fidel, 2015). Information obtained were signalment, history, physical examination, clinicopathological testing (complete blood count, biochemistry profile and serum total

T4), and abdominal ultrasound. Mild to moderately compromised cats were treated with fenbendazole (5 days), followed by novel protein or hydrolysed elimination diet for at least 14 days to rule out parasitism and food response diarrhoea, respectively. Afterwards, endoscopy or FTB were obtained. Otherwise, severely compromised patients were submitted to intestinal biopsy after blood works and ultrasound. Biopsies were obtained by laparotomy (duodenum, jejunum and/or ileum) or endoscopy (duodenum). Stomach and colonic biopsies were not considered in this study. All patients were negative to retroviral screening. Cats with extra-gastrointestinal diseases or comorbidities were excluded.

Chronic enteropathy activity index

The feline chronic enteropathy activity index (FCEAI) was calculated in all cases (Jergens et al., 2010). This score is composed by gastrointestinal signs (vomiting, diarrhoea, anorexia, weight loss, lethargy; each sign received 0-3 points according to severity), hyperproteinaemia (yes = 1 point, no = 0 point), hypophosphataemia (yes = 1 point, no = 0 point), increased serum ALT and/or ALP activities (yes= 1, no= 0 point). Endoscopic lesions parameter was not included because FTB were obtained in most cats. A questionnaire was filled by the owners at the first visit or phone calls. A composite score was subsequently calculated yielding values for mild (2 to 5), moderate (6 to 11) and severe (12 or greater) enteropathy (Bailey et al., 2010).

Histopathological classification

Biopsy samples were fixed in neutral-buffered formalin and embedded in paraffin wax. Tissue were sectioned at 3 µm and stained with haematoxylin and eosin. All sections were reviewed by a single board-certified pathologist (AR) who was blinded to the clinical information. Published diagnostic algorithm was used to differentiate LGAL from IBD (Kiupel et al., 2011).

Biopsies from the control and IBD groups were evaluated according to the WSAVA Gastrointestinal Standardization Group template (Day et al., 2008).24 This template assesses the duodenal (villous stunting, epithelial injury, crypt distension, lacteal dilation and mucosal fibrosis), and inflammation changes (intraepithelial lymphocytes and LP lymphocytes, eosinophils, neutrophils and plasma cells). They were scored as absent= 0, mild= 1, moderate= 2, or severe= 3. Finally, histological severity scores were recorded and determined to be normal (WSAVA score 0), mild (1-6), moderate (7-13), severe (14-20), and very severe (>20) (Procoli et al, 2013). Jejunal and ileal biopsies were scored according to the duodenal biopsy template (Casamian-Sorrosal et al., 2010). Only cats with lymphoplasmacytic enteritis were included.

Modified WSAVA score was used for LGAL cases that included morphological features (villous stunting, epithelial injury and crypt distension) applied for duodenum, jejunum and ileum (Maunder et al., 2012). These features were scored as absent= 0,

mild= 1, moderate= 2, or severe= 3. Total scores were classified as normal (score= 0), mild (1-3), moderate (4-6), severe (7-9), and very severe (>10) according to a calculated proportion of the classification mentioned above.

Lymphoma was classified according to the National Cancer Institute working formulation. Only cats with T cell LGAL were included. This type of lymphoma presented 0 to 5 mitoses/high-power field (hpf) and a nuclear size <1.5X the size of a red blood cell (Valli et al., 2011). CD3 and CD20 immunophenotyping was performed in all LGAL and IBD cases with severe lymphoplasmacytic inflammation as previously described (Kiupel et al., 2011).

For statistical evaluation, the small intestinal segment with the higher template score of each individual was considered.

P-gp immunohistochemistry

The primary monoclonal antibody C494 (Calbiochem, Merck KGaA, Darmstadt) was used for P-gp detection (1:200) as previously reported (Van der Heyden et al., 2009; Van der Heyden et al., 2011a; Van der Heyden et al., 2011b). As secondary antibody, biotinylated goat anti-mouse serum was used (1:200) (Dako, Glostrup, Denmark). StreptABComplex/HRP (Dako, Glostrup, Denmark) was applied for immunolabelling (Van der Heyden et al., 2009; Van der Heyden et al., 2011a; Miyoshi et al., 2002). Sections of feline liver and adrenal gland were used as positive controls

(Figure 1A) (Van der Heyden et al., 2009; Van der Heyden et al., 2011a). Primary antibody was substituted for a non-immune serum as negative control. All samples were batched and run together in May 2013. Repeats of the same intestinal samples to gauge positive and negative staining were performed. All staining sections were reviewed by a board-certified pathologist (AR) who was blinded to the clinical information and diagnoses.

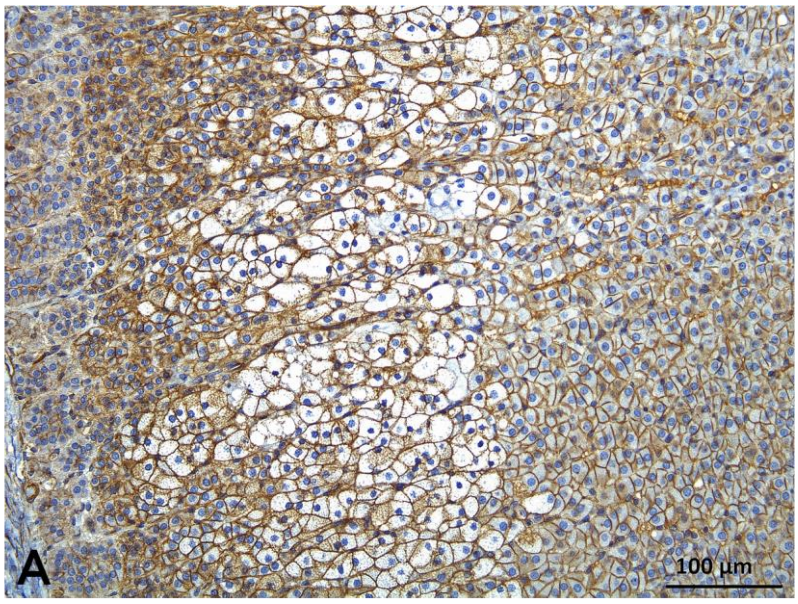

Figure 1. (A) Adrenal cortex as P-gp positive control with marked intensity (brown) of cell margins in reticular zona fasciculata and reticularis; scale bar, 100 µm.

Epithelial P-gp immunolabelling was scored according to a previous semi-quantitative scoring system described in canine IBD by Van der Heysen et al. (2011b): 0, no epithelial P-gp expression; 1, discontinuous P-gp expression at the apical border; and 2, continuous P-gp labelling at the apical border of the epithelium was considered as normal (Figure 2A and B). Continuous P-gp immunoexpression was considered as normal according to Van der Heysen et al. (2009). The immunointensity of the positive control tissue was considered marked.

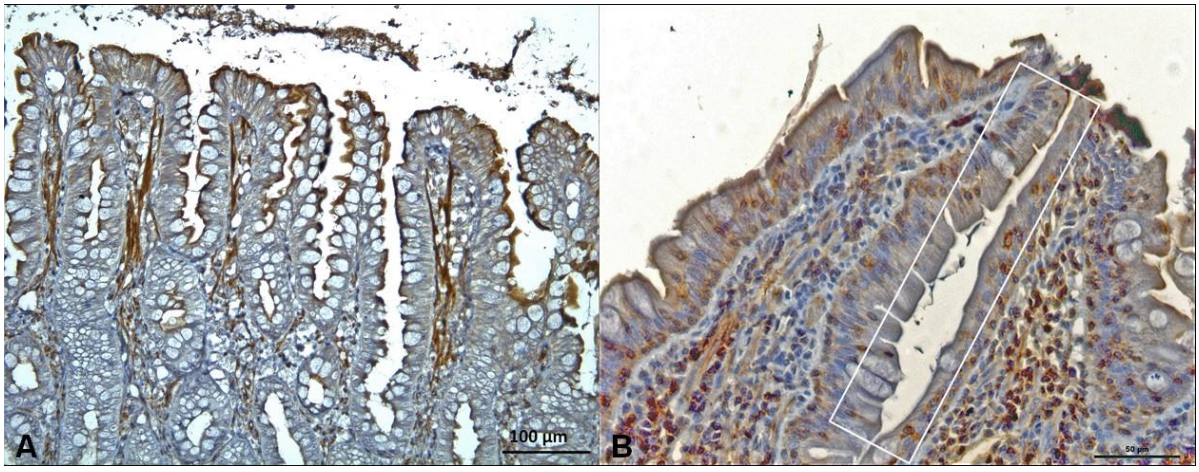

Figure 2. (A) Continuous epithelial P-gp immunoexpression at the apical membrane of colonocyte from the colon of a control cat (score 2); scale bar, 100 µm. **(B)** Non-continuous epithelial P-gp immunoexpression at the apical membrane of enterocytes (white quadrate) and continuous expression in the remaining epithelium with marked intensity of lymphocytes at the lamina propria from the jejunum of a cat with IBD (score 1); scale bar, 50 µm.

Lymphocytes and neoplastic cells at LP immunolabelling were evaluated by a semi-quantitative assessment which included immunostaining intensity and grade (percentage of positive cells). Intensity was scored as 0= negative; 1= weak; 2= moderate; and 3= marked (Figure 3A, B, C and D). The immunointensity of the positive control tissue was considered marked. The grade was defined as 1= <25% positive cells; 2= 25-50% positive cells; and 3= >50% (Miyoshi et al., 2002; Lee et al., 2007). One hundred cells in each of 10 hpf were counted at random and the average was calculated. The overall expression level was calculated multiplying the intensity with percentage and classified as weak (1-2), moderate (3-5) and marked (6-9).

Statistical analysis

Analysis was performed by SPSS statistics software (SPSS 22.0 version, Chicago, IL, USA) adopting a level of significance (p) of <0.05. Shapiro-Wilk test were used for test normality of the data. Non-parametric tests were applied for parameters that did not present a normal distribution, and median and range were used for data description. Kruskal-Wallis test and Mann-Whitney U-test were used to compare continuous variables and χ^2 test for non-continuous variables. Correlations between all variables were evaluated using Spearman's rank correlation test (ρ).

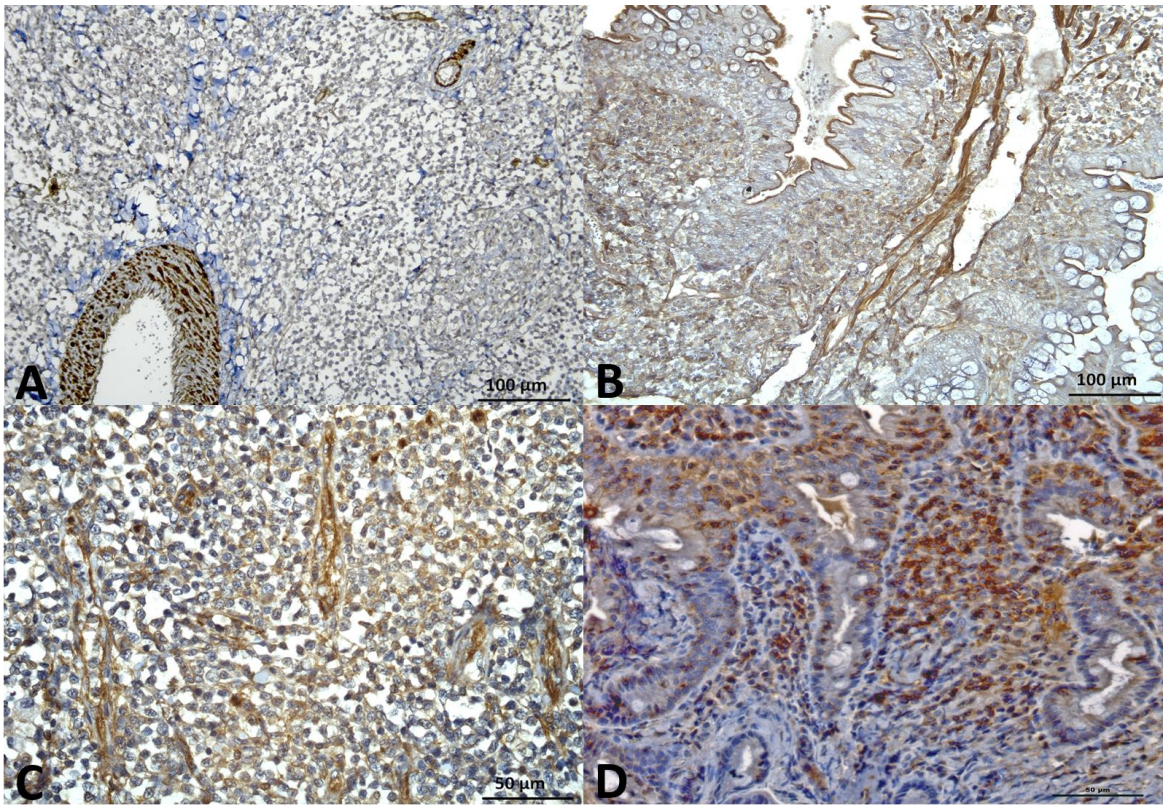

Figure 3. (A) No P-gp expression of the tumour cells at the lamina propria of a low grade alimentary lymphoma (intensity 0); scale bar, 100 µm. **(B)** Weak expression of the neoplastic cells at the lamina propria from ileum in a cat with inflammatory bowel disease (intensity 1) and marked expression of the muscular fibers at the LP; scale bar, 100 µm. **(C)** Moderate expression of the tumour cells at the lamina propria in a cat with low grade alimentary lymphoma (intensity 2); scale bar, 50 µm. **(D)** Marked expression of the tumour cells at the lamina propria (black arrows) from the jejunum of a cat with inflammatory bowel disease (intensity 3); scale bar, 50 µm.

RESULTS

A total of 19 cats met the inclusion criteria, 9 were IBD and 10 were LGAL cases. The IBD group was younger (median= 5, range 2-12 years; p= 0.005) than LGAL group (median= 12.5, range 9-15 years). Despite the cats with IBD showed a higher median body weight (median= 4.38, range 2.00-7.00 kg, p= 0.778) than LGAL group (median= 3.86, range 2.20-6.26 kg), no statistically difference was detected.

Population was composed of 13 male and 6 female cats. Four males (2 neutered and 2 intact) and 5 spayed female cats were included in the IBD group, and 9 neutered male and 1 spayed female cats in the LGAL group. Breeds represented in the IBD group were Domestic Shorthair (DSH, 3), Domestic Longhair (2), Siamese (2), Persian (1) and Norwegian Forest (1) cats. Only DSH cats were represented in the LGAL group.

Concerning FCEAI, the IBD and LGAL group obtained the same median score (median= 11; range IBD= 4-11 and range LGAL= 5-14) that corresponded to moderate enteropathy (Table 1). However, when patients are grouped in composed score a significant difference was observed between groups because most of the cats with IBD presented a mild (22%) and moderate (67%) FCEAI scores whereas cats with LGAL showed more frequently a moderate (60%) and severe (30%) activity index but there is overlapping between the groups (χ^2= 28.83; p= 0.025).

Table 1. FCEAI, modified and total WSAVA scores and P-glycoprotein immunoexpression of Control, IBD and LGAL group.

Group	FCEAI	Modified WSAVA score	Total WSAVA score
Control (median)	**-**	**0**	**1[a]**
IBD (median)	**11[a]**	**2[a]**	**6[b]**
LGAL (median)	**11[b]**	**6[b]**	**-**

FCEAI: feline chronic enteropathy activity index; WSAVA: world small animal veterinary association; LP: lamina propria; IBD: inflammatory bowel disease; LGAL: low grade alimentary lymphoma; -: non-score. Different letters show a significant difference ($p< 0.05$).

In the IBD group, endoscopy biopsies were obtained from 3 cats and FTB from 6 cats. Samples were collected mostly from the duodenum. In the LGAL group, FTB were obtained in all cats except 1 patient. Lymphoma was mostly diagnosed in jejunum (6 cats out of 10), followed by duodenum (2) and ileum (2).

All HCC and 2 SC presented with mild lymphoplasmacytic infiltration according to the WSAVA scoring. Nonetheless, a significant difference was observed between the control and IBD group with this latter presenting higher scores ($p= 0.001$; Table 1). Moreover, modified WSAVA scoring that only included the morphological features of the WSAVA template presented with a significant difference between LGAL and IBD group ($\chi^2= 9.32$; $p< 0.001$; Table 1). Ninety percent of cats with LGAL showed moderate to severe histological score according to the composed modified WSAVA whereas most of the cats with IBD (67%) presented with a mild score. Lineal correlation was found between FCEAI and total WSAVA, FCEAI and modified

WSAVA scores, and total WSAVA and modified WSAVA (Spearman's $\rho > 0.746$; $p < 0.001$).

Control group showed a strong and continuous epithelial P-gp immunolabelling at the apical membrane of the enterocytes (duodenum, jejunum and ileum). Intensity seemed to increase from the base of the crypts to the tips of the villi. Moreover, more than 50 percent of the intraepithelial lymphocytes population expressed marked immunolabelling in the small intestine. None cats of the control group showed immunostaining in the LP.

Concerning to P-gp expression in the epithelium, all cats of the control group and 56% of the patients with IBD presented a continuous or normal expression in the epithelium whereas 80% of the cats with LGAL showed a discontinuous or abnormal immunoexpression in the epithelium ($\chi^2 = 11.52$; $p = 0.003$; Figure 4). When contingency table was applied, the LGAL group showed frequently a discontinuous epithelial expression compared to the other groups within abnormal P-gp immunoexpression ($p < 0.003$; Figure 4).

Regarding P-gp expression in the LP, all cats from the control group did not show immunoexpression. On the other hand, lower number of the cats with IBD presented with marked immunolabelling (44%) in the inflammatory cells of the LP compared to cats with LGAL (60%) where tumour cells showed immunolabelling.

Moreover, moderate immunolabelling in the LP was observed in 22% of the cats with IBD.

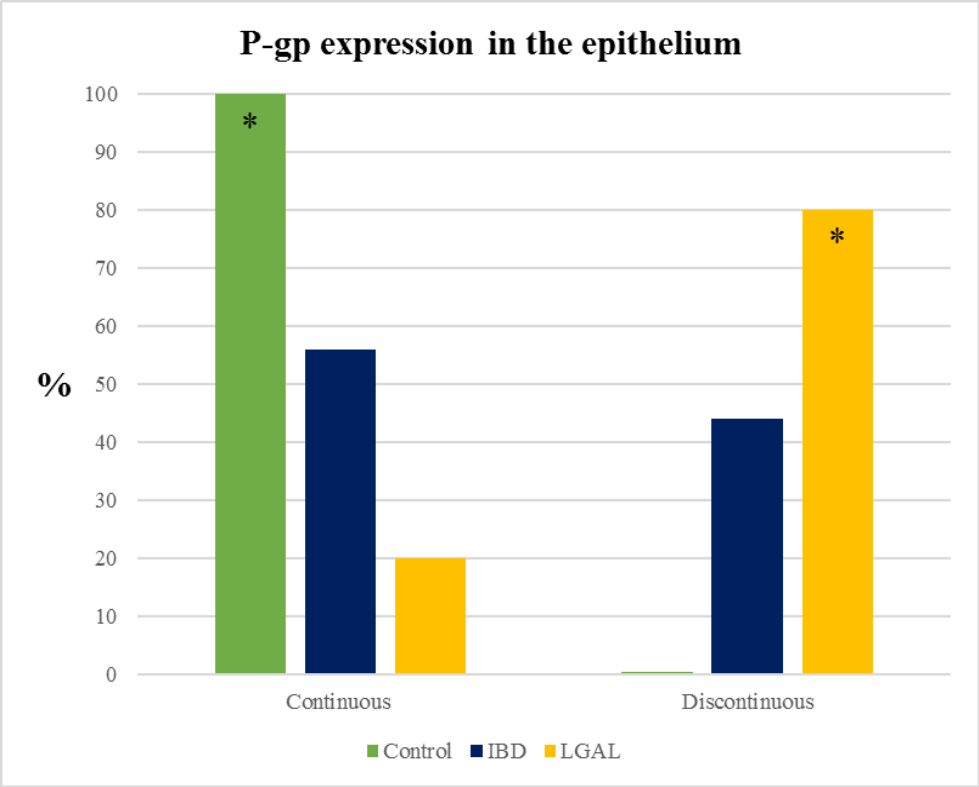

Figure 4. Percentage of cats of the control, IBD and LGAL group with continuous (normal) or discontinuous P-gp expression in the intestinal epithelium (χ^2). P-gp: P-glycoprotein; IBD: inflammatory bowel disease; LGAL: low grade alimentary lymphoma; %: percentage. *: significant difference ($p< 0.05$) of frequency in comparison with the other groups within continuous and discontinuous epithelial expression.

However, 34% of cats with IBD and 30% with LGAL did not show expression in the LP (χ^2= 15.31; *p*= 0.018; Figure 7). When contingency table was applied, when only moderate P-gp expression in the LP was considered, the IBD group was

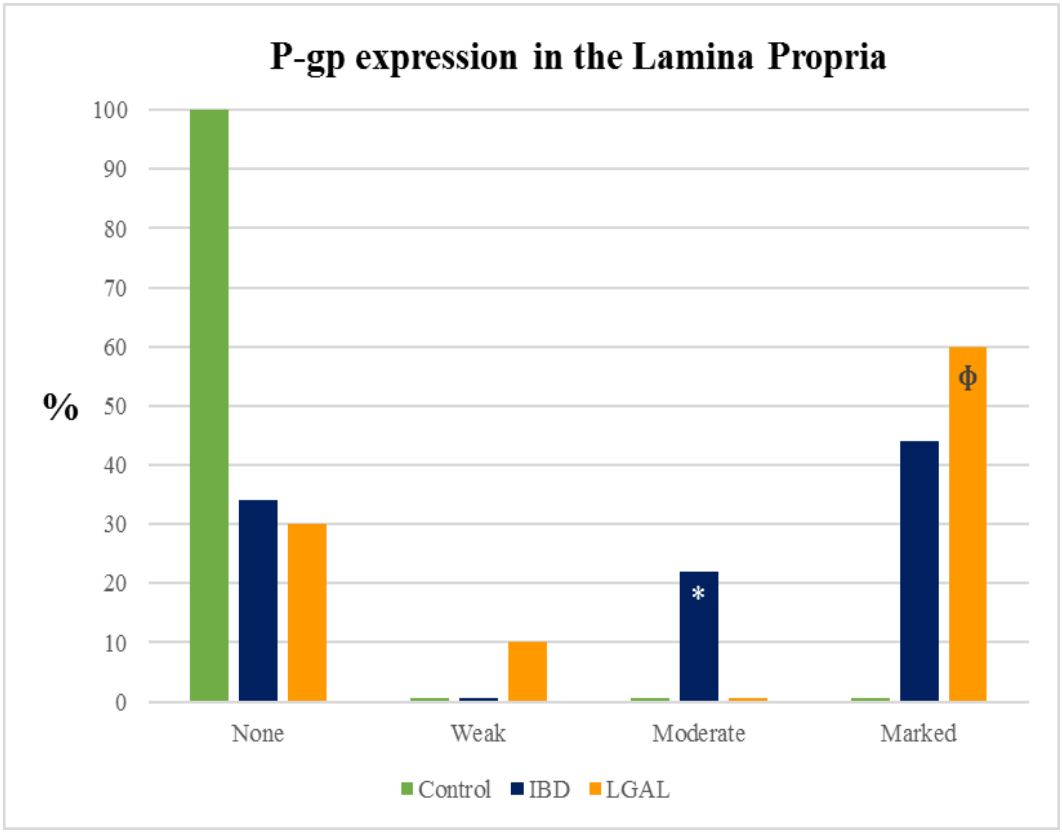

Figure 5. Percentage of cats of the control, IBD and LGAL group with different grades of P-gp expression in the intestinal lamina propria (χ^2). P-gp: P-glycoprotein; IBD: inflammatory bowel disease; LGAL: low grade alimentary lymphoma; %: percentage. *: significant difference (*p*< 0.05) of frequency in comparison with the other groups within moderate expression in the LP. ϕ: tendency (p= 0.05) of frequency compared to the other groups within marked expression in the LP.

significantly more frequent distributed in this expression compared to the other groups (p= 0.03; Figure 5). In addition, cats with LGAL tended to show more frequently a marked expression within this type of immunolabeling in the LP (p= 0.05; Figure 5).

No correlation was observed between P-gp immunostaining in the epithelium and FCEAI, total and modified WSAVA scores (Spearman's $\rho < -0.447$). Association was observed between P-gp expression in the LP and FCEAI, P-gp expression in the LP with total and modified WSAVA scores as well (Spearman's $\rho > 0.594$; $p < 0.001$). Negative correlation was observed between P-gp immunoexpression in the epithelium and immunolabelling in the LP (Spearman's $\rho = -0.660$; $p < 0.001$).

DISCUSSION

The population of animals used in the present study confirmed previous findings showing that cats with IBD are younger and tend to have a higher body weight compared to cats with LGAL though overlap was present. Male cats were overrepresented in LGAL group as well as DSH cats in both studied groups in agreement to previous reports (Norsworthy et al., 2013; Kiupel et al., 2011; Maunder et al., 2012; Waly et al., 2005; Scott et al., 2011).

FCEAI serves to define disease activity in cats with IBD and scores obtained herein are similar to those previously reported (Jergens et al., 2010). Furthermore, this study shows that FCEAI might be used in LGAL. Median FCEAI score was the same

for both groups, but most of the cats with LGAL showed moderate to severe activity index that may be due to lymphoma is a more severe disease.

In the present study, IBD was located mainly in duodenum and T cell LGAL in jejunum confirming previous reports (Barrs and Beatty, 2012a; Norsworthy et al., 2013; Briscoe et al., 2011; Kiupel et al., 2011; Miyoshi et al., 2002; Daniaux et al., 2014; Moore et al., 2012; Lingard et al., 2009). One limitation of this study is that most of the biopsies of the cats with IBD was only obtained from the duodenum as well as the biopsies of the cats with LGAL were more frequently obtained from jejunum. Therefore, these conclusions should be carefully considered.

According to the WSAVA scoring, mild or moderate enteritis are more frequently described in cats with IBD coinciding with our findings (Briscoe et al., 2011; Maunder et al., 2012; Daniaux et al., 2014; Marsilio et al., 2011). Unexpectedly, all cats of the HCC group showed mild inflammation without clinical signs and no morphological changes; this finding is supported by previous studies where control and asymptomatic cats had intestinal mild inflammation (Nguyen et al., 2006; Fragkou et al. 2016).

According our findings, a previous study found correlation between the WSAVA template and the FCEAI (Jergens et al., 2010). This correlation was observed despite pancreatitis and hypocobalaminaemia was not completely ruled out.

Furthermore, the WSAVA score was used with FTB that it did not influence in the correlation result.

Maunder et al. (2012) showed severe duodenal changes in LGAL applying modified WSAVA, but herein was found moderate ones. Despite LGAL group showed frequently severe modified WSAVA score than cats with IBD in the present study and correlation between FCEAI and modified WSAVA was observer, further studies are needed to determinate whether this modified template might help to differentiate between chronic enteropathies.

To our knowledge, this is the first report about P-gp expression in the intestinal epithelium and LP of cats with IBD and LGAL. Epithelial P-gp expression is normal in small and large intestine of cats and humans as our study, but in dogs is only expressed in colon (Van der Heyden et al., 2009; Cordon-Cardo et al., 1990; Van der Heyden et al., 2011b). The discontinuity or loss of the epithelial P-gp expression in cats with IBD in the present study may be a cause or consequence. As cause, mutant mice lacking P-gp expression (mrd1a-/-) spontaneously develop colitis due to a defect in barrier function may increase accumulation of bacterial breakdown products within epithelial cells and/or immunological dysregulation of cytokines and chemokines in the intestine (Wilk et al., 2005). Otherwise as consequence, all morphological and inflammatory alterations destroy the intestinal epithelium or may reduce the P-gp production and expression like occur in rats (Buyse et al., 2005). However, these hypotheses would not

be implicated in all cases of IBD since 56% of cats presented with normal expression of P-gp in the epithelium. Furthermore, 80% of the LGAL cases presented with an abnormal discontinuous epithelial P-gp expression likely secondary to epitheliotropism of the tumour or as consequence mechanism explained above (Buyse et al., 2005; Kiupel et al., 2011). The results obtained in this study may reflect that epithelial P-gp expression may not have a key role in the feline IBD pathogenesis but it might be in LGAL, or cats with IBD have an adaptive mechanism to maintain their epithelial P-gp expression. Further studies are needed in cats to elucidate these questions.

P-gp expression in the LP is absent in healthy humans, cats and dogs coinciding with the present study (Van der Heyden et al., 2009; Cordon-Cardo et al., 1990; Allenspach et al., 2006). However, dogs with IBD can present P-gp overexpression in the inflammatory cells are infiltrating in the LP, and tumour cells of the canine and feline lymphoma (Brenn et al., 2008; Allenspach et al., 2006; Bergman et al., 1996; Ginn et al., 1996). In the present study, the IBD group showed a P-gp expression in the LP similar to the dogs with steroid responsive IBD (Allenspach et al., 2006). Otherwise, the LGAL group showed a marked immunolabelling more frequently in the LP compared to cats with IBD that it may be associated with the severity of the diseases. Nonetheless, 30% of cats with LGAL did not show P-gp expression in the LP. In canine high-grade lymphoma, 67% of cases not present P-gp expression but the positive cases present marked expression as the present study (Lee et al., 1996). Further studies

evaluating immunohistochemistry or molecular expressions are needed to obtain conclusions and elucidate its role in the pathogenesis, severity, resistance to treatment and prognosis.

FCEAI, modified and total WSAVA scoring did not show a statistically significant correlation with P-gp expression of the epithelium, but did with the LP likely because more severe clinical signs and higher histological scores may indicate a more severe intestinal affectation in the LP. This severity may be reflected with higher expression of P-gp in the LP that might be related with the treatment response and prognosis of IBD and LGAL, but further studies are needed to confirm this hypothesis. In addition, the results of the present study agree with data reported in dogs with IBD where correlation between WSAVA scores and epithelial P-gp expression was not found (Van der Heyden et al., 2011b). Nonetheless, epithelial P-gp immunolabelling score used in the present study was the same reported previously in dogs. Maybe, it would be needed a new semi-quantitative system score to carry out the statistical analysis. Negative correlation between P-gp expression in the LP and epithelium is likely due to the severity of the histological lesions affect to the epithelium as well as LP since an abnormal or discontinuous epithelial immunostaining presented higher immunolabelling in the LP.

This study presented limitations, most of the cases were recruited retrospectively and FCEAI was calculated by record data or owner interview by phone calls, therefore

subjectivity may be an uncontrolled variable. Although intraobserver variation among histopathologic evaluations of intestinal tissues were not present due to one pathologist evaluated all biopsies, an intraobserver variation could have existed (Willard et al., 2002). Cases of triaditis were not included, however feline specific pancreatic lipase was not available in all cases and pancreatitis was ruled out by ultrasound. Moreover, all histopathological diagnosis was made prior to the availability of polymerase chain reaction for antigen receptor rearrangements; thereby a misdiagnosis could have occurred. However, FTB was available in almost all cats and immunohistochemistry was performed by increasing the sensitivity and specificity (Kiupel et al., 2011). Prognostic value of the P-gp expression in IBD and LGAL was not studied because the follow-up from all cases was unavailable and it was not an objective of the present study. Further studies are needed to evaluate the P-gp as prognostic marker in IBD and AL in cats.

CONCLUSION

Most of cats with LGAL presented with a higher FCEAI and modified WSAVA as well as discontinuous P-gp immunoexpression in the intestinal epithelium and marked immunostaining in the LP in comparison with cats with IBD. Nonetheless, 66% of cats with IBD showed P-gp immunolabelling in the LP and correlation between expression and histopathological alterations was observed. Therefore, P-gp expression

might be implicated in the pathogenesis, severity, resistance to treatment and prognosis of both diseases and further studies are needed to elucidate these conclusions.

4.2 STUDY II

Cyclooxygenase-2 immunoexpression in intestinal epithelium and lamina propria of cats with inflammatory bowel disease and low grade alimentary lymphoma

J. Castro-López, A. Ramis, M. Planellas, M. Teles and J. Pastor (Under review)

ABSTRACT

Objective: Cyclooxygenase 2 (COX-2) is an inducible isoform by cellular activation, proinflammatory cytokines and growth factors. The aims of the current study were to evaluate COX-2 immunoexpression in epithelial and lamina propria (LP) of cats with inflammatory bowel disease (IBD) and low grade alimentary lymphoma (LGAL), as well as to correlate them with clinical signs and histopathological scoring.

Method: Cats diagnosed with IBD and LGAL (2007-2013) were included in the current study. Feline chronic enteropathy activity index (FCEAI) was calculated for all cases. Control group was composed by 3 healthy indoor cats and 5 sick cats died or were euthanized (non-gastrointestinal illness). Diagnosis and classification of IBD and LGAL was established according to the WSAVA gastrointestinal standardization group template and the National Cancer Institute formulation, respectively. Furthermore, a modified WSAVA template was applied for LGAL evaluation. Immunolabelling for COX-2 (polyclonal rabbit anti-murine antibody) was performed on biopsy samples. Epithelial and LP (inflammatory or neoplastic cells) COX-2 immunolabelling was calculated according to the grade and intensity. The most representative segment scored by the WSAVA and the modified WSAVA were used for statistical analysis.

Results: Significant difference was found regarding COX-2 intensity overexpression in the epithelial cells of IBD and LGAL groups when compared to control cats, but not

between the groups of sick cats, whereas no differences were found regarding the grade of immunoreactivity between groups. No difference was found for COX-2 immunoexpression at the LP between all groups. However, 3 cats from LGAL group showed COX-2 expression in neoplastic cells at the LP. There were no correlations between epithelial or LP COX-2 expression and FCEAI and histological alterations.

Conclusions: Increased COX-2 intensity at the epithelial cells observed in cats with IBD and LGAL may be secondary to the inflammatory response or a protective function in the intestinal reparation. COX-2 expression at the LP was presented in 33% of LGAL. This result provides a reason for further investigation concerning the role of COX-2 expression in feline alimentary lymphoma.

INTRODUCTION

Inflammatory bowel disease (IBD) and low grade alimentary lymphoma (LGAL) are common causes of chronic enteropathies (CEs) in cats (Richter et al., 2003; Jergens et al., 2010; Guilford et al., 2011; Barrs and Beatty, 2012a; Jergens, 2012; Norsworthy et al., 2013). IBD is a chronic immune-mediated disease whose cause remains unknown but is likely multifactorial (Jergens et al., 2010; Guilford et al., 2011; Jergens, 2012; Norsworthy et al., 2013). Currently, alimentary lymphoma (AL) is the most common anatomic form of lymphoma and its cause is also unknown (Vail et al., 1998; Bertone et al., 2002; Louwerens et al., 2005; Milner et al., 2005; Lingard et al., 2009; Stützer et al., 2011; Barrs and Beatty, 2012a;). IBD and LGAL can affect any segments of the gastrointestinal (GI) tract and clinical differentiation between them may be a challenge. Therefore, histopathological diagnosis is always needed though overlapping may also occur, complicating the definitive diagnosis (Moore et al., 2005; Briscoe et al., 2011; Kiupel et al., 2011; Barrs and Beatty, 2012a; Barrs and Beatty, 2012b; Jergens, 2012). In addition, evolution from chronic intestinal inflammation to AL has been proposed in cats but definitive proof is lacking (Mahony et al., 1995; Louwerens et al., 2005).

Cyclooxygenase 2 (COX-2) is an inducible inflammatory regulator isoform by cellular activation, proinflammatory cytokines, growth factors, tumour promoters and prostaglandin mediator (Vane et al., 1998; Williams et al., 1999; Yu et al., 2007; Ghosh

et al., 2010). Prostaglandin E_2, a COX-2 metabolite, has many biological roles including mediating pain, modulation of cytokine production, induction of regulators of angiogenesis, production of proinflammatory mediators and promotes tumourigenesis (Funk, 2001; Charlier et al., 2003). Furthermore, overexpression of COX-2 may be a consequence of inflammation leading to increased levels of Bcl-2 and resistance to apoptosis of the cells, thus enhancing the risk of cancer (Tsujii et al., 1998; Sakamoto et al., 2005). To the author's knowledge, there is only one available study in cats that included 6 cases of intestinal lymphoma and described negative COX-2 immunoexpression (Beam et al., 2003), and there is no study describing COX-2 immunoexpression in feline IBD and LGAL.

The aim of the present study was to evaluate COX-2 immunoexpression at the epithelium and lamina propria (LP) of cats with IBD and LGAL. The second objective was to correlate the COX-2 immunolabelling with clinical signs and histopathological scoring.

METHODS

Study population

Control group was composed of 3 healthy control indoor female cats (HCC, median age= 2 years; range= 1-5 years) owned by the personal staff were submitted to endoscopy prior to ovariohysterectomy and duodenal biopsies were obtained, and 5 sick

cats (SC, median age= 7 years; range= 1-18 years) who died or were euthanized for unrelated GI diseases and full thickness biopsies (FTB) from duodenum, jejunum and ileum were obtained within 1 hour. Cats had not received glucocorticoids (GC), chemotherapy, non-steroidal anti-inflammatory drugs (NSAIDs) or antibiotics with immunomodulatory action such as doxycycline and azithromycin previously. All these cats were recruited from the Fundació Hospital Clínic Veterinari of the Universitat Autònoma de Barcelona.

Approval consent was signed and accepted by the owners and procedures were approved by the Ethical Committee from the Faculty of Veterinary Medicine and Bioscience Engineering of Universitat Autònoma de Barcelona (CEAAH 2354).

IBD and LGAL cases of the study were collected between 2007 and 2013 from the Fundació Hospital Clínic Veterinari of the Universitat Autònoma de Barcelona. The inclusion criteria were the presence of chronic GI signs (>3 weeks duration), complete medical history and no previous GC, chemotherapy, NSAIDs or antibiotics with immunomodulatory action treatments six months before the presentation. Information obtained from all cats included signalment (age, breed, sex, body weight), history, physical examination, clinicopathological testing (complete blood count, biochemistry profile and total T4 and abdominal ultrasonography). All patients were negative to feline leukaemia virus antigen and immunodeficiency virus antibodies. Cats with mild to moderate clinical signs were treated at the beginning with antiparasitic for 5 days,

followed by elimination diet (novel protein or hydrolysed elimination diets) for at least 14 days to rule out parasitism and food response enteropathy, respectively. Posteriorly, endoscopy or FTB were obtained. Otherwise, severely compromised patients were submitted to intestinal biopsy after blood works and ultrasonography. These patients did not receive antiparasitics or placed on diet trials at presentation, but did during treatment in cats with IBD. Biopsies were obtained by laparotomy (duodenum, jejunum and/or ileum) or endoscopy (duodenum). Stomach and colonic biopsies were not considered in this study. Cats with extra-GI diseases were excluded from the study.

Chronic enteropathy activity index

The feline chronic enteropathy activity index (FCEAI) was applied to all studied cats (Jergens et al., 2010). This index gave a scoring to GI signs (vomiting, diarrhoea, anorexia, weight loss, lethargy; 0 to 3 points for each sign according to severity), hyperproteinaemia (yes = 1 point, no = 0 point), hypophosphataemia (yes = 1 point, no = 0 point), increased serum alanine aminotransferase (ALT) and/or alkaline phosphatase (ALP) activities (yes = 1 point, no = 0 point). Endoscopic lesions parameter was not included because FTB were performed in most of the cats and endoscopy was not repeated. A questionnaire was filled by the owners at the first visit or phone calls. A composite score was subsequently calculated yielding values for mild (2 to 5), moderate (6 to 11) and severe (12 or greater) CE (Bailey et al., 2010).

Histopathological classification

Biopsy samples were fixed in neutral-buffered formalin and embedded in paraffin wax. Tissue was sectioned (3 µm) and stained with haematoxylin and eosin. Single board-certified pathologist (AR) reviewed all sections and was blinded to the clinical information. Previously published diagnostic algorithm was used to differentiate IBD from LGAL (Kiupel et al., 2011).

Biopsies from the control and IBD groups were evaluated according to the world small animal veterinary association (WSAVA) GI Standardization Group template (Day et al., 2008). This template only assesses the duodenal morphological features (villous stunting, epithelial injury, crypt distension, lacteal dilation and mucosal fibrosis) and inflammation changes (intraepithelial lymphocytes, LP lymphocytes and plasma cells, eosinophils, neutrophils, other cells) from the duodenum. They were scored as absent= 0, mild= 1, moderate= 2, or severe= 3. Finally, histologic severity scores were recorded and determined to be normal (score 0), mild (1-6), moderate (7-13), severe (14-20), and very severe (>20) (Procoli et al., 2013). Jejunal and ileal biopsies were scored according to the WSAVA template as Casamian-Sorrosal et al. (2010) described in these segments.

Modified WSAVA score was used for LGAL cases that included morphological features (villous stunting, epithelial injury and crypt distension) and applied to

duodenum, jejunum and ileum (Maunder et al., 2012). These features were scored as absent= 0, mild= 1, moderate= 2, or severe= 3. Total scores were classified as normal (score= 0), mild (1-3), moderate (4-6), severe (7-9), and very severe (>10) according to a calculated proportion of the classification mentioned above.

LGAL cases were classified according to the National Cancer Institute working formulation. The number of mitoses between 0-5 at high-power field and small nuclear size (<1.5X the size of a red blood cell) correspond to LGAL (Valli et al., 2011). Furthermore, CD3 and CD20 immunophenotyping was performed in LGAL and severe IBD cases as previously described (Kiupel et al., 2011).

For statistical evaluation, the small intestinal segment with the higher or modified histological score of each individual was considered.

COX-2 immunohistochemistry

Sections (3 µm) were routinely deparaffinised, rehydrated and antigen retrieval at pH 6 was performed by PT-Link Automatic System (Dako Glostup, Denmark). Immunostaining was performed on a Dako Autostainer Plus, using procedures, buffers and solutions provided by the manufacturer. Primary antibody binding was detected with a standard two-layer indirect method (EnVision; DakoCytomation). Chromogen staining was developed with diaminobenzidine. Slides were counterstained with haematoxylin. The primary antibody (polyclonal rabbit anti-murine COX-2; Cayman

Chemical, Ann Arbor, Michigan, USA) at a 1 in 500 dilution was used. A rabbit polyclonal antibody against *Leishmania infantum*, kindly provided by Instituto de Salud Carlos III (Madrid, Spain), was used for negative control purposes (1:3000). Sections of feline foetal kidney (Figure 1) and cutaneous squamous cell carcinoma were used as positive controls (Hayes et al., 2006; Newman et al., 2006; Bardagí et al., 2012). COX-2 immunohistochemical staining was performed on a normal feline lymph node as a negative control.

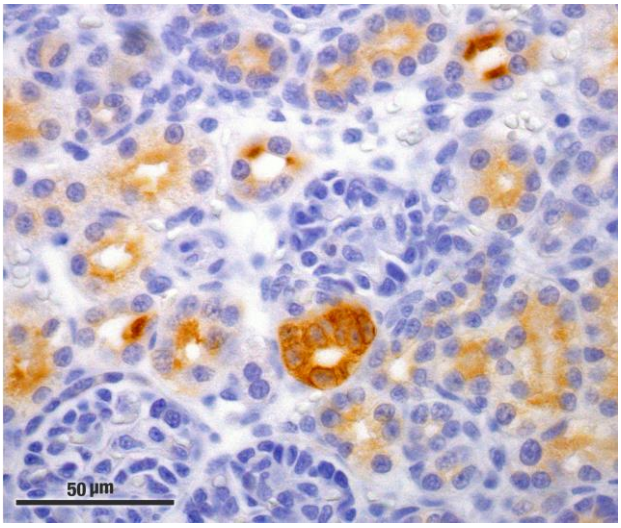

Figure 1. Macula densa from a foetal kidney showing marked intensity of COX-2 immunoexpression and apical border of renal tubular cells expressing moderate intensity.

Epithelial, inflammatory and/or neoplastic cells COX-2 immunolabelling was evaluated by a semi-quantitative assessment which included staining grade (percentage

of positive cells) and intensity. Five 10X fields from each slide were evaluated. The grade (percentage) was evaluated by the following scoring system: 0= negative; 1= <10% of cells staining positive; 2= 10-30%; 3= 31-60%; 4= >60%. Intensity was evaluated by the following scoring system: 0= negative; 1= weak staining; 2= moderately intense staining; and 3= marked intense staining. Intensity of positive control cells was considered marked staining (Beam et al., 2003). The final expression score was calculated multiplying the intensity with percentage and classified as weak (1-2), moderate (3-5), marked (6-8) and very marked (>9).

Statistical analysis

Statistical analysis was performed using SPSS statistics software (SPSS 17.0 version, Chicago, IL, USA) adopting a level of significance of $p< 0.05$. Shapiro-Wilk test were used for tested normality of the data. Non-parametric tests were applied for data that did not present a normal distribution, and median and range were used for summary. The Kruskal-Wallis test was used to compare continuous variables (FCEAI, WSAVA and modified WSAVA scores, epithelial and LP COX-2 expression) between groups. The Mann-Whitney test was used as post-test analysis for the evaluation of the variation between the different groups.

RESULTS

A total of 28 cats met the inclusion criteria but 8 cats were eliminated because biopsy samples were unavailable. Therefore, 11 cats with IBD and 9 cats with LGAL were studied. The median age was 5 years (range= 2-12) for IBD group and 12 years (range= 8-15) for the LGAL group. LGAL group presented a slightly higher body weight (median= 4.2 kg; range= 3.00-6.26) than IBD group (median= 3.88 kg; range= 2.00-6.00). All cats were neutered, except 1 intact female and 1 intact male from the IBD group. There were 5 (45%) female and 6 (55%) male cats in the IBD group and 1 (11%) female and 8 (89%) male cats in the LGAL group. Breeds represented in the IBD group were Domestic Shorthair (DSH, 4), Domestic Longhair (3), Siamese (2), Persian (1) and Norwegian Forest (1) cats. All cats belonging to the LGAL group were DSH cats.

Endoscopy biopsies were obtained from 3 cats and FTB from 8 cats of the IBD group. Samples were obtained mostly from the duodenum (9 cats). Regarding the inflammatory cells infiltration at the LP, 8 cases had lymphoplasmacytic (73%) and 3 eosinophilic (27%) inflammation. FTB were collected in all cats with LGAL except for 1 patient. All LGAL animals were T cell lymphoma and it was most commonly diagnosed in the jejunum (6 cats out of 9), followed by duodenum (2) and ileum (1).

Median of FCEAI score obtained by LGAL group was 11 (range= 5-14) and IBD group was 9 (range= 4-12) corresponding to moderate CE, but no statistical significant difference was found (p= 1.000; Table 1).

Table 1. FCEAI, modified and total WSAVA scores and COX-2 immunoexpression of Control, IBD and LGAL group.

Group	FCEAI	Modified WSAVA score	Total WSAVA score	Intensity Epithelium	% Epithelium	Total Epithelium	Intensity LP	% LP	Total LP
Control (median)	-	0^a	1^a	2^a	4^a	8^a	0^a	0^a	0^a
IBD (median)	9^a	1^a	5^b	3^b	4^a	12^a	0^a	0^a	0^a
LGAL (median)	11^a	2^b	-	3^b	4^a	12^a	0^a	0^a	0^a

FCEAI: feline chronic enteropathy activity index; WSAVA: world small animal veterinary association; %: percentage; LP: lamina propria; IBD: inflammatory bowel disease; LGAL: low grade alimentary lymphoma; -: non-score. Different letters show a significant difference (p< 0.05).

According to the WSAVA template, IBD group showed a significant statistically higher score of morphological and inflammatory changes compared to the control group (p= 0.011, Table 1 and Figure 2). Considering the modified WSAVA score, that only includes the morphological features of the WSAVA template, LGAL group presented a significantly higher value than the IBD (p= 0.011) and control group (p< 0.001, Table 1 and Figure 2). No significant difference was found between IBD and control group according to the modified WSAVA score (p= 0.156, Table 1 and Figure 2). No lineal correlation was found between FCEAI and total WSAVA, and modified WSAVA scores (p> 0.05).

COX-2 epithelial immunoexpression was observed in all studied cats, except 3 SC that belong to the control group. Regarding the intensity of expression, 82% of cats with IBD (9 out of 11) and 67% with LGAL (6 out of 9 cats) presented a marked inten-

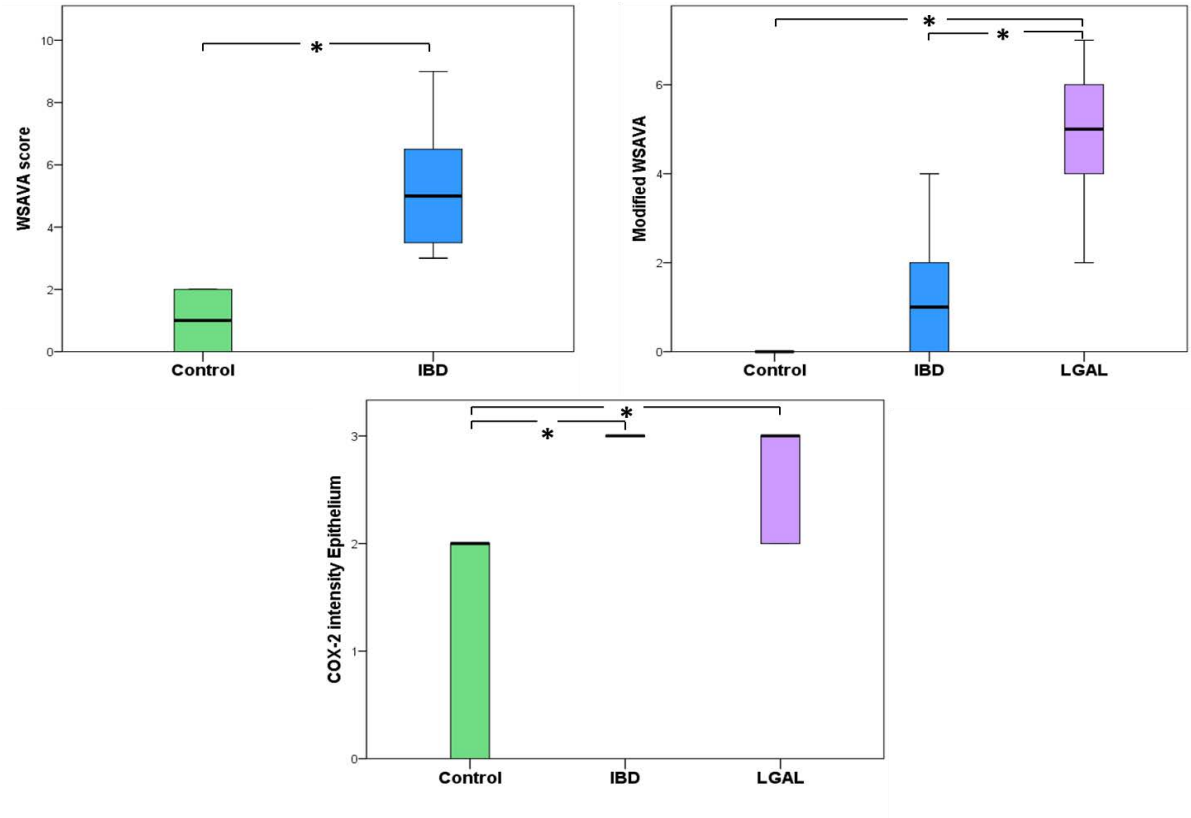

Figure 2. WSAVA scores comparison between Control and IBD group. Symbol (p< 0.05): * between Control and IBD group. Modified WSAVA scores comparison between Control, IBD and LGAL group. Symbol (p< 0.05): * between Control and LGAL group; between IBD and LGAL group. COX-2 intensity in the epithelium comparison between Control, IBD and LGAL group. Symbol (p< 0.05): * between Control and IBD group; between Control and LGAL group. Box plots represent median, 25th percentil, 75th percentil. maximum and minimum. WSAVA: world small animal veterinary association; IBD: inflammatory bowel disease; LGAL: low grade alimentary lymphoma; COX-2: cyclooxygenase 2.

sity; remaining cats presented a moderate intensity. No significant difference was detected between these groups (p= 1.000, Table 1). Sixty-three per cent of cats from the control group showed a moderate epithelial COX-2 intensity, but the other ones did not present staining as mentioned above. Furthermore, control group presented lower intensity in comparison with the IBD (p= 0.001) and LGAL group (p= 0.008, Table 1 and Figure 2). Regarding the percentage of cells, all cats from the IBD, 67% (6 out of 9) from the LGAL and 63% (5 out of 8) from the control group showed immunolabelling in more than 60% of the enterocytes, and no statistically significant difference was observed concerning to staining grade (p= 0.081, Table 1). COX-2 immunoexpressions are presented in Figure 3A, B, C and D.

COX-2 expression at the LP was absent in all cats from the control and IBD group (Table 1). In the LGAL group, 2 cats presented moderate intensity and 1 cat a marked intensity immunolabelling of neoplastic, however the immunoreactivity was presented in less than 10% of cells (Table 1 and Figure 4A, B and C). Regardless, no statistical significant differences were observed according to intensity, staining grade and final score of COX-2 expression at the LP between the three groups (p> 0.05, Table 1).

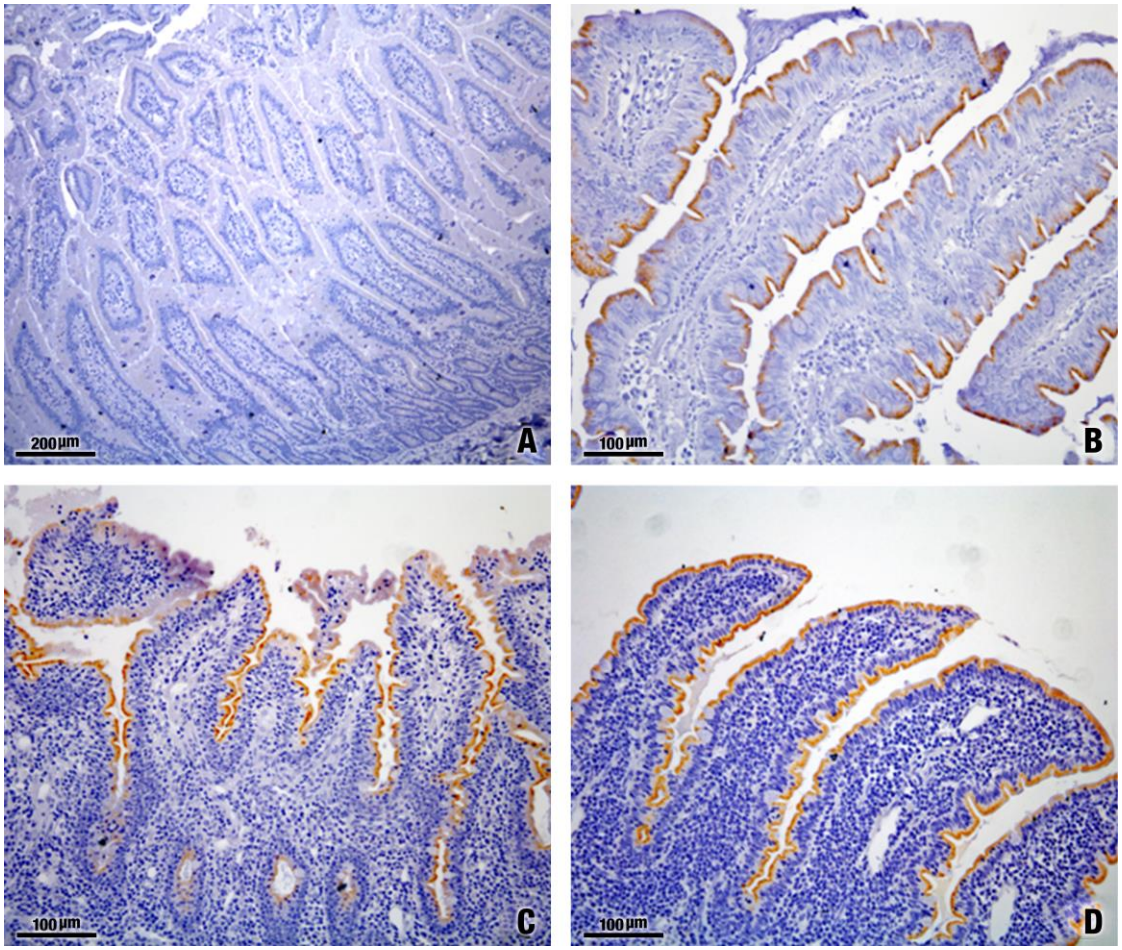

Figure 3. (A) Absence of COX-2 immunolabelling in the apical membrane of the epithelium from duodenum of a sick cat from the control group (score 0); scale bar, 200 µm. **(B)** Moderate epithelial COX-2 immunoexpression of the apical membrane of enterocytes from the duodenum of healthy control cats (score 2); scale bar, 100 µm. **(C)** Marked epithelial COX-2 labelling of the apical membrane of enterocytes from the jejunum of severe lymphoplasmacytic enteritis (score 3); scale bar, 100 µm. **(D)** Marked epithelial COX-2 labelling of the apical membrane of enterocytes from the jejunum of severe lymphoplasmacytic enteritis (score 3); scale bar, 100 µm.

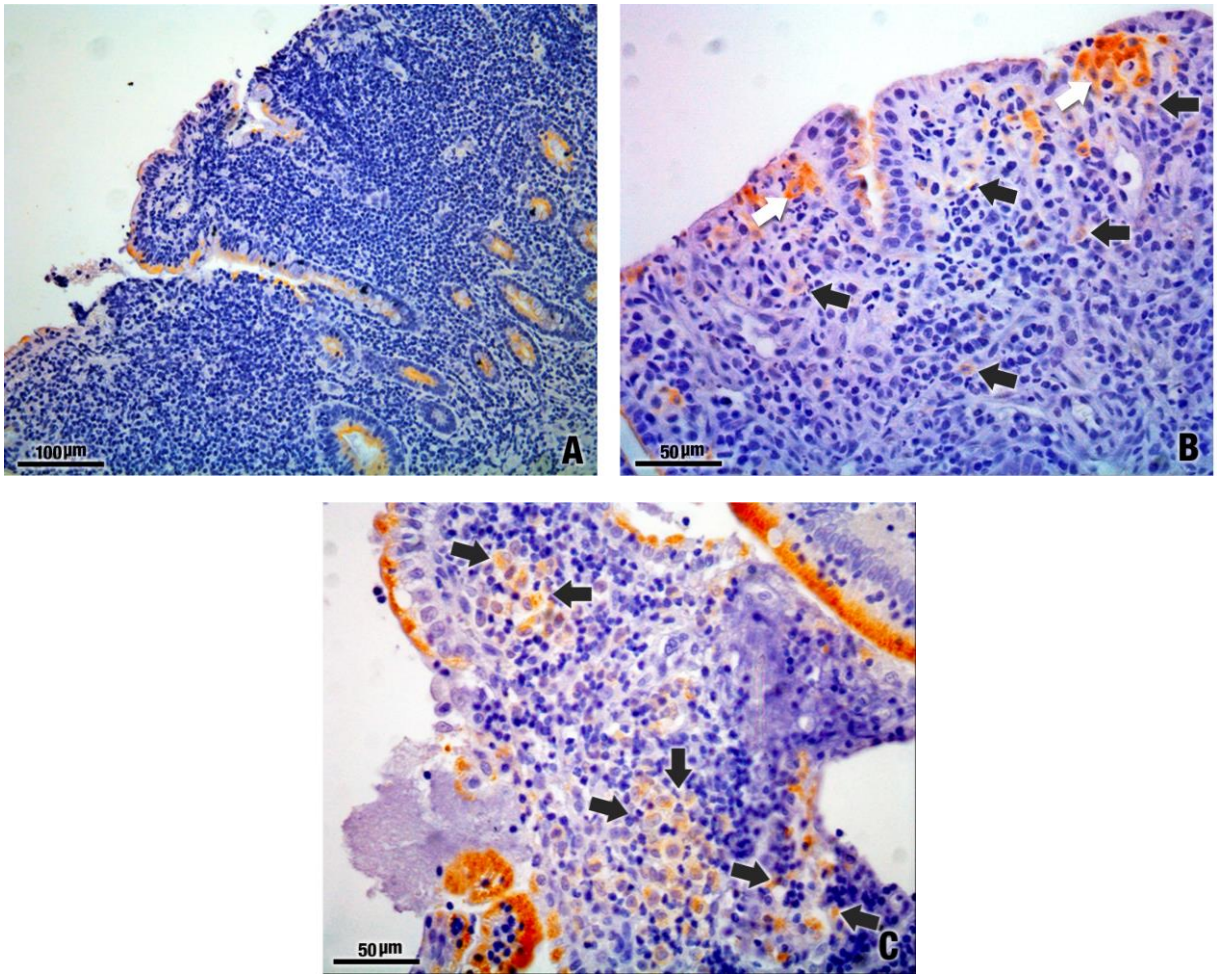

Figure 4. (A) No expression of COX-2 at the neoplastic lymphocytes at the lamina propria of a low grade alimentary lymphoma (intensity 0); scale bar, 100 µm. **(B)** Moderate COX-2 expression of a few neoplastic cells at the lamina propria (black arrows) and enterocytes with marked reactivity (white arrows) from a cat with low grade alimentary lymphoma (intensity 2); scale bar, 50 µm. **(C)** Marked expression of COX-2 at some neoplastic lymphocytes at the lamina propria (black arrows) in a cat with low grade alimentary lymphoma (intensity 3); scale bar, 100 µm.

Statistically significant lineal correlations were not observed between epithelial or LP COX-2 expression and FCEAI and histological alterations ($p > 0.05$; Spearman's $\rho < 0.354$).

DISCUSSION

The population of animals used in the present study confirmed previous findings showing that IBD affects younger cats compared to AL, although overlap was present. Male cats were overrepresented in LGAL group as well as DSH cats in both studied groups in agreement to previous reports (Guilford et al., 2001; Richter, 2003; Jergens et al., 2010; Kiupel et al., 2011; Barrs and Beatty, 2012a; Jergens, 2012; Maunder et al., 2012; Norsworthy et al., 2013).

Lymphoplasmacytic inflammation has been the most common inflammatory pattern defined in cats with IBD and was localized most frequently in duodenum (Briscoe et al., 2011; Norsworthy et al., 2013; Daniaux et al., 2014). Duodenum is the most common GI segment evaluated, but it is unlikely that IBD is restricted to this segment. This location is probably overrepresented due to limitations of endoscopy to obtain samples from lower small intestine segments. Furthermore, FTBs are likely more obtained from the duodenum as well than the jejunum and ileum like the present study. According to previous reports, T cell LGAL was more frequently localized in the

jejunum (Lingard et al., 2009; Kiupel et al., 2011; Barrs and Beatty, 2012a; Moore et al., 2012; Norsworthy et al., 2013).

In contrast to our findings, a study in cats found correlation between the WSAVA template and the FCEAI, however no correlation was observed in studies performed in dogs (Guilford et al., 2001; Allenspach et al., 2007; Jergens et al., 2010; Procoli et al., 2013). These discrepancies might be due to the FCEAI was calculated retrospectively in most of the cats. Furthermore, pancreatitis and hypocobalaminaemia that could worsen clinical signs, was not completely ruled out. Also, in the present study we used FTB from different intestinal segments that have been evaluated by a single pathologist, which might have influenced WSAVA scores.

Maunder et al. (2012) observed severe duodenal morphological changes applying the modified WSAVA scoring in LGAL and herein moderate changes were found. Nevertheless, a significant difference was observed between LGAL and IBD group in the present study regarding modified WSAVA scoring. Further studies are needed to determinate whether this histological scoring might help to differentiate between CE.

To our knowledge, this is the first report regarding COX-2 expression in the intestinal epithelium and LP of cats with IBD and LGAL. COX-2 is classically considered an inducible enzyme, but it is also considered a constitutive enzyme

expressed in the GI tract (Kefalakes et al., 2009). Moreover, COX-2 products might be involved in maintaining the integrity of intestinal mucosa (Kefalakes et al., 2009). Differences between species have been described about epithelial COX-2 expression along the GI tract in normal individuals. COX-2 is expressed in the ileocoecal junction and colon in rodents, in all the GI tract in dogs and in the stomach and colon in humans (Jackson et al., 2000; Porcher et al., 2004; Wilson et al., 2004; Haworth et al., 2005; Paiotti et al., 2007; Romero et al., 2008; Amorim et al., 2014; Dumusc et al., 2014). In the present study, cats of the control group presented epithelial COX-2 expression in duodenum, jejunum and ileum (data not shown). Therefore, this supports the need of more studies to clarify COX-2 expression and role in normal individuals.

Regarding immunoreactivity in healthy feline GI tract, only one study described COX-2 immunoexpression in basal granulated cells of the epithelium using a polyclonal antiprostaglandin H synthetase-2 (COX-2) human C terminus antibody (Satoh et al., 2013). Some differences may be found depending on the antibody used, in our study, immunolabelling was found in the cytoplasm of the enterocytes in 5 cats of the control group (3 HCC and 2 SC). The discordance on inmunoexpression may be explained by the different anti-reagent used, or different affinity of the antibody, however the antibody used herein was previously used in cats (Singer et al., 1998; Jackson et al., 2000; Joo et al., 2002; Joo et al., 2003; Hayes et al., 2006; Newman et al., 2006; Paiotti et al., 2007; Bardagí et al., 2012). The presence of COX-2 positive and negative

enterocytes in SC of the control group might be explained by the degree of epithelial autolysis in the samples. However, SC were necropsied within 1 hour. Even though epithelial autolysis was not observed in the histopathology, molecular autolysis cannot be totally ruled out that could influence on the COX-2 expression. Another possible explanation is the individual variability, it has been demonstrated that only 50 to 80% of healthy humans presents COX-2 expression in colon and stomach (Jackson et al., 2000; Paiotti et al., 2007; Romero et al., 2008; Dai et al., 2015). Further studies with larger number of cats are needed to obtain conclusions about normal COX-2 expression in the GI tract.

Epithelial intensity immunoexpression in IBD and LGAL groups was significantly higher in comparison with control group though no statistical difference was found between the group of cats with IBD and LGAL. Higher epithelial COX-2 immunolabelling has been reported in humans with gastritis induced by *Helicobacter pylori*, ulcerative colitis or Crohn's disease compared to normal epithelium. These observations agree with the present study (Singer et al., 1998; Jackson et al., 2000; Paiotti et al., 2007; Romero et al., 2008; Dai et al., 2015). The increased COX-2 expression may be due to GI epithelial ulceration, however in our study only 2 cats with LGAL presented epithelial ulceration (data not shown) (Singer et al., 1998; Jackson et al., 2000). Furthermore, it has been described that COX-2 expression increases after feeding in feline duodenum, but this is unlikely since the cats used in the present study

were fasted for anaesthetic procedure or were anorectics (Satoh et al., 2013). Increased mucosal levels of prostaglandin E_2 in humans and interleukin-1β in dogs with IBD and food responsive diarrhoea have been linked to an increased COX-2 immunoexpression or upregulation (Singer et al., 1998; Dumusc et al., 2014). Based on these studies, it has been suggested that cytokines and prostaglandins induced by an inflammatory response increase COX-2 in the intestinal mucosa as a protective mechanism (Singer et al., 1998; Dumusc et al., 2014). Regarding LP, no expression was found in any cat from control or IBD groups. At the same time, the normal feline lymph node did not present COX-2 expression (data not shown), as previously described in dogs (Rodrigues et al., 2011). In humans with IBD, macrophages and polymorphs are stained by COX-2 at the LP (Singer et al., 1998; Roberts et al., 2001; Paiotti et al., 2007; Romero et al., 2008; Dai et al., 2015). However, those inflammatory cells are not present in feline IBD, and probably for this reason immunolabelling was not found in our cases. Association between COX-2 upregulation and development of lymphoma, as occurs in some tumours, remains unknown but COX-2 overexpression is associated with cell proliferation and angiogenesis (Joo et al., 2002; Joo et al., 2003; Mohammed et al., 2004; Ohsawa et al., 2006). In this study, only 3 cats with LGAL presented COX-2 expression in lymphoid tumour cells. Conversely, Beam et al. (2003) did not find COX-2 immunoexpression in 6 cats with AL. This disagreement may be due to a different immunohistochemical technique. A recent report stated that 15% of canine lymphoma

presented COX-2 overexpression which agrees with the present findings (Asproni et al., 2014). However, other studies in canine lymphoma did not find COX-2 immunoreactivity (Mohammed et al., 2004; Rodrigues et al., 2011). Furthermore, studies in humans revealed that most of non-Hodgkin's lymphoma (>50% of cases) had COX-2 expression by tumour cells (Hazar et al., 2006; Paydas et al., 2007; Ma et al., 2012). Thus, COX-2 upregulation in lymphomas has been associated with the aggressiveness, relapsed, worst response to therapy and less overall survival (Hazar et al., 2006; Paydas et al., 2007; Sugita et al., 2007; Ma et al., 2012). This latter could not be determined in our study because not all cats had available follow-up. Prospective studies are needed in cats with different lymphoma phenotypes and anatomical locations to further understand the role of COX-2 in feline AL. No correlations were observed between FCEAI, histological alterations, IBD and LGAL with COX-2 expression. Similar results have been obtained in canine IBD, and human lymphoma and IBD (Hazar et al., 2006; Paiotti et al., 2007; Paydas et al., 2007; Ma et al., 2012; Dumusc et al., 2014).

This study presented some limitations, most of the cases were recruited retrospectively and FCEAI was calculated by record data or owner interview by phone calls, therefore subjectivity may be an uncontrolled variable. Although intraobserver variation among histopathologic evaluations of intestinal tissues were not present due to one pathologist evaluated all biopsies, an intraobserver variation could have existed

(Willard et al., 2002). Cases of triaditis were not included, however feline specific pancreatic lipase was not available in all cases and pancreatitis was ruled out by ultrasound. Moreover, all histopathological diagnosis was made prior to the availability of polymerase chain reaction for antigen receptor rearrangements; thereby a misdiagnosis could have occurred. However, FTB was available in almost all cats and immunohistochemistry was performed by increasing the sensitivity and specificity (Kiupel et al., 2011).

CONCLUSION

Increased COX-2 intensity at the epithelial cells observed in cats with IBD and LGAL may be secondary to the inflammatory response or a protective function in the intestinal reparation. COX-2 expression at the LP was presented in only 33% of LGAL cats, thus further investigation of COX-2 expression in feline LGAL are needed to clarify its importance in tumourgenesis, prognostic and response to therapy.

4.3 STUDY III

Pilot study: duodenal Mdr-1 and Cox-2 gene expression in cats with inflammatory bowel disease and low grade alimentary lymphoma

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ABSTRACT

Objectives: Multidrug resistance 1 (*MDR-1*) encodes a protein called P-glycoprotein (P-gp) that serves as an efflux pump membrane protein implicated in intestinal homeostasis and drug resistance. Cyclooxygenase-2 (COX-2) is a key enzyme in the synthesis of proinflammatory prostaglandins, tumourigenesis and in mucosal defence. Despite the importance of MDR-1 and COX-2, changes in their mRNA levels have not been studied in cats with inflammatory bowel disease (IBD) and low grade alimentary lymphoma (LGAL). The present study aimed to determine the mRNA levels of *Mdr-1* and *Cox-2* in cats with IBD and LGAL, and to evaluate their correlation with clinical signs, histological severity and between genes.

Methods: Cats diagnosed with IBD (n= 20) and LGAL (n= 9) between 2008 and 2015 were included in the current study. Three healthy animals composed the healthy control cats group that endoscopy was performed immediately before to the ovariohysterectomy. All duodenal biopsy samples were obtained by endoscopy. Feline chronic enteropathy activity index was calculated for all cases. IBD histopathology was classified according to severity. *Mdr-1* and *Cox-2* mRNA levels were determined by absolute reverse transcriptase-quantitative real time PCR.

Results: Statistically significant differences were observed for *Mdr-1* and *Cox-2* mRNA levels between the IBD and LGAL groups. No correlations were observed between molecular expression for genes, FCEAI and histological grading for IBD, and between

Mdr-1 and *Cox-2* gene. However, a positive statistically significant correlation was observed between *Mdr-1* and *Cox-2* expression in the duodenum of cats.

Conclusions: *Mdr-1* and *Cox-2* gene expression is increased in cats with LGAL compared to cats with IBD. The control group tended to have lower values than both diseased groups. These results suggest that these genes may be involved in the pathogenesis of IBD or LGAL in cats.

INTRODUCTION

Inflammatory bowel disease (IBD) and low grade alimentary lymphoma (LGAL) are common causes of chronic enteropathies (CEs) in cats (Guilford et al., 2001; Richter, 2003; Jergens et al., 2010; Barrs and Beatty, 2012a; Jergens, 2012; Norsworthy et al., 2013). The aetiology of IBD remains largely unknown, but multifactorial mechanisms are hypothesised to contribute to the pathogenesis (Guilford et al., 2001; Jergens et al., 2010; Jergens, 2012; Norsworthy et al., 2013). On the other hand, LGAL is the most common form of lymphoma in cats and its cause is also unknown (Vail et al., 1998; Bertone et al., 2002; Louwerens et al., 2005; Milner et al., 2005; Moore et al., 2005; Lingard et al., 2009; Briscoe et al., 2011; Kiupel et al., 2011; Stutzer et al., 2011; Barrs and Beatty, 2012a; Barrs and Beatty, 2012b). Additionally, progression of IBD to lymphoma has been proposed in cats and humans but no direct link has been established (Mahony et al., 1995; Louwerens et al., 2005).

In humans, the multidrug resistance 1 (*MDR-1*) or *ABCB-1* gene belongs to the family of ABC (ATP-binding cassette)-transporters gene (Fromm, 2002; Ho et al., 2003; Annese et al., 2006). *MDR-1* gene codes for P-glycoprotein (P-gp), an efflux pump membrane protein that actively transports substrates such as bacterial products and drugs from the inside to the outside of cells being likely implicated in the IBD pathogenesis and resistance to treatments (Fojo et al., 1987; Thiebaut et al., 1987; Ho et

al., 2003; Annese et al., 2006;). Previously, it has been described that *Mdr-1a*-deficient mice develop colitis and that human patients with IBD show reduced expression of *MDR-1* in the colon (Panwala et al., 1998; Langmann et al., 2004). Furthermore, elevated P-gp expression levels have been shown in canine IBD and has been associated with poor prognosis (Allenspach et al., 2006). Likewise, *Mdr-1* has been implicated in treatment resistance to various chemotherapy regimens used in veterinary species (Mealey, 2012).

Cyclooxygenase 2 (COX-2) is an inducible inflammatory regulator isoform by cellular activation, proinflammatory cytokines, growth factors, tumour promoters and prostaglandin mediator in humans and mice (Vane et al., 1998; Williams et al., 1999; Yu et al., 2007; Ghosh et al; 2010). Moreover, their metabolites have many biological roles such as prostaglandin E_2 that is a mediator of pain, modulator of cytokine production, inducer of regulators of angiogenesis, producer of proinflammatory mediators and promoter of tumourigenesis (Funk, 2001; Charlier and Michaux, 2003). Additionally, overexpression of COX-2 may be a consequence of inflammation leading to increased levels of bcl-2 protein and resistance to apoptosis of cells, and thus enhancing the risk of cancer (Tsujii et al., 1998; Sakamoto et al., 2005). Lately, COX-2 has been also considered a constitutive enzyme expressed in the gastrointestinal tract that acts as part of the mucosal defence mediated by prostaglandin and leading to reduced severity of colitis (Wallace, 2001; Kefalakes et al., 2009).

An *in vitro* study has shown that the expression and activity of P-gp can be modulated by COX-2 (Patel et al., 2002). However, this association has not yet been reported in human IBD (Østergaard et al., 2009). Furthermore, a positive correlation between COX-2 and the ABC-transporter immunoexpression has been described in different human neoplasms and non-Hodgkin's lymphoma (Li et al., 2007; Szczuraszek et al., 2009).

Since an inflammatory response in the mucosa may be affected by higher exposure from a defective intestinal barrier, the aims of the present study were: 1) to determine the mRNA transcription levels of *Mdr-1* and *Cox-2* in cats with IBD and LGAL and; 2) to correlate these values with clinical signs and histological severity, and to evaluate whether there is a correlation between *Mdr-1* and *Cox-2* genes.

MATERIAL AND METHODS

Study population

Thirty-one cats were included in this retrospective study. The electronic database of the Royal Veterinary College of London and Veterinary Teaching Hospital of the Universitat Autònoma de Barcelona (UAB) was searched between June 2008 and May 2015. Cats with signs of CE and duodenal biopsy samples available in RNAlater® (Sigma, Saint Louis, Missouri) were identified. The inclusion criteria were the presence

of gastrointestinal signs (>3 weeks duration), complete medical record and not having received any glucocorticosteroids or non-steroidal anti-inflammatory drugs in the previous 3 months. Information obtained included signalment, history, physical examination, clinicopathological testing (haematology, serum biochemistry, and total T4), fecal parasitology and abdominal ultrasonography. Serum cyanocobalamin, folate and specific feline pancreatic lipase immunoreactivity (fPLI) were included if available. Retroviral status of all cats was negative.

Feline CE activity index (FCEAI) was retrospectively calculated for all cats (Jergens et al., 2010). This scoring is composed of gastrointestinal signs (vomiting, diarrhoea, anorexia, weight loss, lethargy; each sign received 0-3 points), abnormal total protein concentration (yes= 1, no= 0 point), hypophosphataemia (yes= 1, no= 0 point), increased serum ALT and/or ALP activities (yes=1, no=0 point) and endoscopic lesions (yes=1, no=0 point). A composite score was subsequently calculated yielding values for mild (2 to 5), moderate (6 to 11) and severe (≥ 12) enteropathy (Bailey et al., 2010).

According to histological diagnosis, the cats were grouped in IBD and LGAL. Biopsies from 3 healthy control female cats (HCC, mean age= 30 months) were taken by endoscopy before ovariohysterectomy which composed the control group. These cats did not present endoscopic abnormalities, gastrointestinal signs and disease.

Approval consent of HCC was signed and accepted by the owners and procedures were approved by the Ethical Committee from the UAB (CEAAH 2354).

Collection of Endoscopic Biopsy Specimen

Samples of duodenal mucosa were collected by endoscopy. A minimum of 6 biopsies from each diseased cat was obtained from the duodenum and fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin. An additional 3 biopsies from each cat were stored in RNA later for 24 hours and after that maintained at -80°C until RNA isolation.

Histopathological classification and immunohistochemistry

For IBD, biopsies were scored according to the criteria of a board-certified pathologist as normal (score 0), mild (1), moderate (2) and severe (3) enteritis based on the architecture features and cellular infiltration of the section. LGAL cases were identified according to the National Cancer Institute working formulation (Vali et al., 2000; Valli et al., 2011). When the lymphoma presented 0 to 5 mitoses at high-power field and a nuclear size <1.5X the size of a red blood cell was classified as LGAL (Vali et al., 2000; Vali et al., 2011).

CD3 and CD79a immunophenotyping was performed in LGAL and severe IBD cases as previously described (Kiupel et al., 2011).

Total RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was individually extracted from duodenum using 500 µL per sample of TRI reagent (Molecular Research Center) and following the manufacturer's instructions. RNA quantification was carried out with a Nanodrop ND-1000 (Thermo Scientific) and RNA quality checked with the Experion RNA StdSens Analysis Kit (BioRad). RNA integrity values obtained were >8, indicative of excellent RNA integrity and quality. Reverse transcription (RT), to generate cDNA, was performed using 1 µg of total RNA, denatured 70 °C, 10 min, Oligo dT_{15}primer (Promega) and SuperScript™ III Reverse Transcriptase enzyme (Invitrogen), in presence of the recombinant ribonuclease inhibitor RNaseOUT™ (Invitrogen) in a final volume of 20 µL. The reaction was performed at 37 °C for 1 h, heat inactivated at 70 °C for 15 min.

Primer design and transcriptional analysis

Primers used for the *Mdr-1* and *Cox-2* gene expression study were designed with Primer3 version 4.0 based on target sequences obtained from *F. catus* database (Table 1). Efficiency of the amplification was determined for each primer pair using serial 10-fold dilutions of pooled cDNA. The efficiency was calculated as E = 10(-1/s) where s is the slope generated from the serial dilutions, when log dilution is plotted against ΔCt (threshold cycle number) (Pfaffl, 2001). The analysis of mRNA levels of target genes,

were assessed with absolute RT-qPCR (reverse transcriptase-quantitative real time PCR).

Table 1. Sequences and efficiencies of primers used for quantitative real-time PCR analysis in duodenum of cats.

Gene name	Acronym	GenBank accession no.	Forward	Reverse	Fragment Size (bp)	Efficiency (%)
ATP-binding cassette carrier B1	***Mdr-1***	GU222365.1	TGGACTGTCGGCTGCTATCT	TGTCCTCCAAAGGCAATCAC	135	101
Cyclooxygenase 2	***Cox-2***	EF036473.1	CCAGGAGGTCTTTGGTCTGG	TGGAACAACCGCTCATCATC	126	96

All reactions were run in the Bio-Rad CFX384 Real-Time PCR Detection System (Bio-Rad Laboratories, USA), according to the protocol: 95 °C for 5 min, 40 cycles of 95 ºC for 30 s, 60 ºC for 30 s. All samples were run in triplicate.

Determination of plasmid copy number in recombinant *E. coli* was used for absolute quantification (Morera et al., 2011). The pGEM easy vector (Promega) with the amplified product of RT-qPCR was transformed into *E. coli* DH5α cells. Bacteria were grown in LB medium overnight and DNA vectors were obtained using Nucleospin Plasmid Quickpure kit (Machery-Nagel), digested with EcoRI to check the insert (Promega) and sequenced with T7 and SP6 primers. The plasmid copy number was determined using the following equation:

$$\text{DNA (copy/µL)} = \frac{6.02\times10^{23}\ \text{(copy/mol)} \times \text{DNA amount (g/L)}}{\text{DNA length (bp)} \times 660\ \text{(g/mol/bp)}}$$

Absolute qPCR was carried out under the same conditions using a 10^7 to 10^1 copies/mL dilution of plasmid DNA (pGEM, Promega). Standard curves (Ct-Threshold cycle *versus* log copy number) were constructed for sample copy number determination.

Statistical analysis

Statistical analysis was performed using SPSS statistics software (SPSS 17.0 version, Chicago, IL, USA) adopting a level of significance of $p< 0.05$. Data were determined being non-parametric, therefore the Kruskal-Wallis test was used to compare continuous variables (FCEAI, histopathological score and genes expression) between groups. Anova test with Tukey post-hoc was used to test differences between IBD and LGAL groups regarding expression of *Cox-2* and *Mdr-1* mRNA. Linear correlations between variables were evaluated using the Pearson correlation coefficient.

RESULTS

A total of 29 cats met the inclusion criteria. According to histological diagnosis, 20 cats were diagnosed with IBD and 9 cats with LGAL. The median age was 119.5 months (range= 30-201) for IBD group whereas for the LGAL group was 140 months (range= 84-205), but statistical difference was not found ($p= 0.248$). Furthermore, IBD group presented a significant higher body weight (median= 4.58 kg, range= 3.20-5.44) than LGAL group (median= 3.20 kg, range= 2.27-4.25; $p= 0.027$). No difference was

observed regarding the body condition score between cats with IBD (median= 4) and LGAL (median= 3; p= 0.246).

All cats in the IBD group were neutered. There were 7 (35%) female and 13 (65%) male cats in the IBD group and in the LGAL group was composed of 6 (67%) female and 3 (33%) male cats. Breeds represented in the IBD group were Domestic Shorthair (DSH, 11), Siamese (5), Domestic Longhair (3), and British shorthair (1) cats. LGAL was represented by DSH (7), Birman (1) and Persian (1) cats.

The most common clinical sign in all cats was weight loss (94%), followed by vomiting (81%), lethargy and inappetence (71%), and diarrhoea (68%). Median FCEAI score in the LGAL group was significantly higher than IBD group (p= 0.030), 11 (range= 5-14) and 9 (range= 5-13), respectively.

Clinicopathological abnormalities detected in this study are summarized in the Table 2.

Concurrent co-morbidities were present in some patients with IBD: 1 cat presented with acute cholangitis, 1 with chronic cholangitis and 1 with chronic bronchitis. fPLI serum concentrations were elevated in 7 cats. Previous co-morbities under treatment were reported in 1 cat with hyperthyroidism and 1 cat with hypercalcaemia. In the LGAL group, co-morbidities were also presented: 1 cat with

chronic kidney disease (CKD), 1 with CKD and hyperthyroidism that was controlled with methimazole treatment, 1 with hepatic lipidosis, 1 with increased fPLI and 1 with hyperthyroidism previously treated with radioiodine therapy.

Table 2. Clinical pathological abnormality in cats with inflammatory bowel disease and low grade alimentary lymphoma.

Parameter ***(range)***	***Clinical-pathohological abnormality***	***IBD*** ***(n affected/total n)***	***LGAL*** ***(n affected /total n)***
Haematocrit (24-45%)	Anaemia	5/21	-
Total protein (61-80 g/l)	Hyperproteinaemia	4/21	-
	Hypoproteinaemia	2/21	4/10
Albumin (28-42 g/l)	Hypoalbuminaemia	4/21	3/10
Globulin (25-46 g/dl)	Hyperglobulinaemia	3/21	-
ALT (25-130 u/l)	Increased	2/21	-
Phosphorus (0.92-2.16 mmol/l)	Decreased	1/21	-
Cobalamin (270-1000 ng/l)	Hypocobalanaemia	2/16	5/10
Folate (9.5-20.2 ng/l)	Increased	2/16	-
	Decreased	2/16	-
fPLI (≥ 5.4µg/l)	Highly suggestive of pancreatitis	7/14	1/5

IBD: inflammatory bowel disease; LGAL: low grade alimentary lymphoma; n: number of cats; ALT: alanine aminotransferase; fPLI: feline pancreatic lipase immunoreactivity (Spec fPL®); -: within of range reference. This table includes only those parameters that showed alterations.

With respect to the inflammatory cells infiltrating the lamina propria, all cats presented with lymphoplasmacytic inflammation, although 3 of the cats had concurrent mild neutrophilic infiltration. Twelve cases corresponded to mild, 6 to moderate and 2 to severe enteritis. All lymphomas were identified as T cell immunophenotype.

According to the gene expression results, cats with LGAL showed statistically significant higher expression levels than cats with IBD for both *Mdr-1* (p= 0.023) and *Cox-2* (p= 0.034) genes (Table 3; Figure 1). In addition, the HCC group showed lower expression values than both groups (Table 3), but this group was not included in the statistical analysis due to the low number of cats.

Table 3. mRNA transcription levels of *Mdr-1* and *Cox-2* in HCC, IBD and LGAL group.

Gene	**HCC**	**IBD**	**LGAL**
***Mdr-1* (mean ± SD)**	1.34E+02 ± 9.40E+01	1.52E+03 ± 1.86E+03	3.60E+03 ± 2.54E+03
***Cox-2* (mean ± SD)**	6.78E+01 ± 3.55E+01	9.66E+01 ± 5.42E+01	2.00E+02 ± 1.45E+02

HCC: healthy control cats; IBD: inflammatory bowel disease; LGAL: low grade alimentary lymphoma; Mdr-1: multidrug resistance 1; Cox-2: cyclooxygenase; SD: standard deviation.

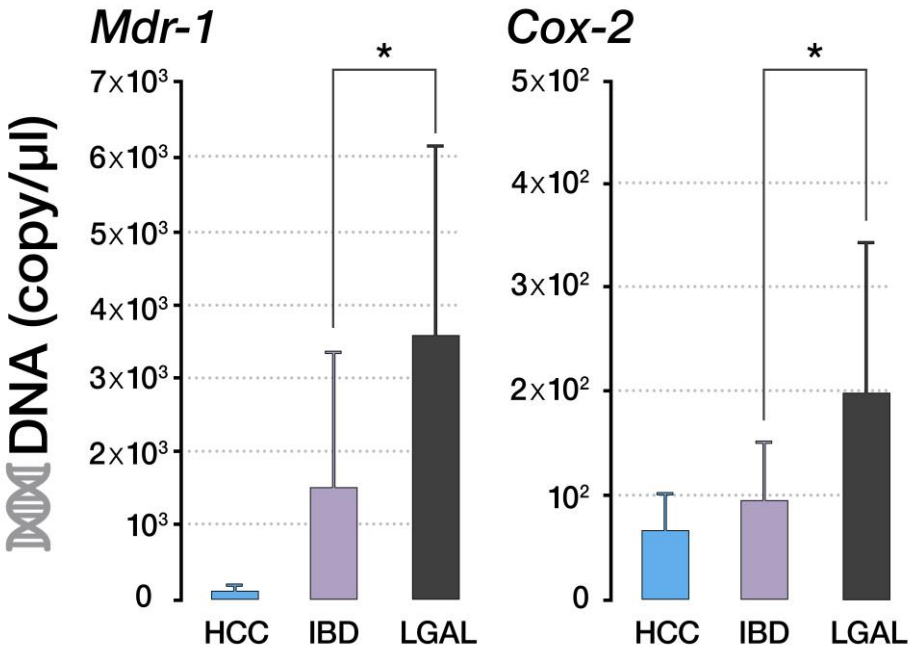

Figure 1. mRNA levels of Mdr-1 and Cox-2 gene in duodenal samples of cats. Analysis of mRNA levels was assessed with absolute RT-qPCR. Values represent the means ± S.E. (n= 3 HCC; n= 20 IBD; n= 9 LGAL group). Statistically significant differences between groups are: LGAL vs. IBD (*p< 0.05). HCC group was not included in the statistical analysis. *Mdr-1*: multidrug resistance 1; *Cox-2*: cyclooxygenase 2; HCC: healthy control cats; IBD: inflammatory bowel disease; LGAL: low grade alimentary lymphoma.

There were no significant correlations detected between FCEAI, histological grade of IBD and either *Cox-2* or *Mdr-1* duodenal gene expression, nor between both genes in the studied groups (p> 0.050; r< 0,344).

DISCUSSION

Results of the present study confirm previous findings that patient with IBD tend to be younger cats and presents with higher body weight compared to LGAL, although a significant overlap was present (Guilford et al., 2001; Richter, 2003; Jergens et al., 2010; Kiupel et al., 2011; Barrs and Beatty, 2012a; Jergens, 2012; Norsworthy et al., 2013; Freiche et al., 2016). Male cats, DSH and Siamese cats were also overrepresented in the IBD group, although a predisposition to this disease has been proposed for the latter breed (Guilford et al., 2001; Richter, 2003; Jergens et al., 2010; Kiupel et al., 2011; Barrs and Beatty, 2012a; Jergens, 2012; Norsworthy et al., 2013; Freiche et al., 2016). Clinical signs described herein are similar to those previously reported (Guilford et al., 2001; Richter, 2003; Jergens et al., 2010; Kiupel et al., 2011; Barrs and Beatty, 2012a; Jergens, 2012; Norsworthy et al., 2013; Fragkou et al., 2016; Freiche et al., 2016).

Cats with IBD from a previous study presented with similar FCEAI scores to those included in the study herein (Jergens et al., 2010). To the author's knowledge, FCEAI has not previously been applied to cats diagnosed with LGAL. A statistically significant difference was found in the FCEAI score between the two diseased groups, likely because LGAL presents with more severe clinical signs and/or chronicity.

Clinicopathological alterations found in this study are similar to previous reports of cats with IBD or LGAL, although none of the cats with LGAL presented with anaemia, which has been reported in other studies (Jergens et al., 2010; Barrs and Beatty, 2012a; Jergens, 2012; Fragkou et al., 2016; Freiche et al., 2016). Furthermore, according to the table 2, hypoproteinaemia, hypoalbuminaemia and hypocobalanaemia were more frequently seen in our LGAL group similar to previous reports (Kiselow et al., 2008; Barrs and Beatty, 2012a).

Co-morbidities were present in several cats in this study. A recent report described that cholangitis is more commonly associated with IBD than triaditis followed by pancreatitis (Fragkou et al., 2016). However, in the present study, fPLI was increased in 50% of cats suffering from IBD compared to only 2 cats with cholangitis confirmed by histology and none of the studied cats presenting with signs of triaditis. Nevertheless, pancreatic or hepatic biopsies were not taken from all patients to ascertain a diagnosis of pancreatitis or cholangitis (Xenoulis, 2015). Other comorbidities (hepatic lipidosis, CKD, hyperthyroidism, chronic bronchial disease) that were present in these cats have been previously reported in medium age to elderly cats (Foster et al., 2004; Caney et al., 2009; Puig et al., 2015; Fragkou et al., 2016).

Consistent with previous reports, lymphoplasmacytic inflammation was the most common inflammatory pattern defined in IBD and T cell immunophenotype in LGAL

cats (Moore et al., 2005; Lingard et al., 2009; Briscoe et al., 2011; Kiupel et al., 2011; Barrs and Beatty, 2012b; Jergens, 2012; Norsworthy et al., 2013; Daniaux et al., 2014).

In the present study, HCC showed only a mild *Mdr-1* expression. Healthy human beings have moderately high *MDR-1* mRNA expression levels in jejunum and colon. In mice, the highest mRNA levels of *Mdr-1* were found in the normal ileum and colon, and these levels gradually decline proximally towards the jejunum, duodenum, and stomach (Fojo et al., 1987; Croop et al., 1989; Nooter and Herweijer, 1991; Chianale et al., 1995). In humans with IBD, greater and differential *MDR-1* mRNA levels have been found in Crohn's disease compared to healthy controls, which is similar to our findings (Yacyshyn et al., 1999). Contrarily, ulcerative colitis has been associated with low expression of the *MDR-1* gene compared to healthy people (Langmann et al., 2004). In the present study, *Mdr-1* mRNA levels tended to be higher in IBD and LGAL compared to HCC, and a statistically significant difference has been observed between the diseased groups. It is not possible to determine if increased *Mdr-1* levels play a role in the aethiopathogenesis of feline IBD or if it is a consequence of the inflammation as previously reported in mice (Wilk et al., 2005). In addition, the higher levels of *Mdr-1* gene observed in the LGAL compared to IBD group could explain the necessity of a more aggressive therapy in the lymphoma. Future studies to determine whether feline *Mdr-1* gene expression is similar to that found in Crohn's disease and to

determine the contribution of this gene to IBD pathogenesis and the implication with resistance to treatment are needed (Annese et al., 2006; Zintzaras, 2012).

To the author's knowledge, there are so far no studies available evaluating *Mdr-1* gene expression comparing LGAL and IBD in cats, since *Mdr-1* gene has only been studied in a feline lymphoma cell line (Okai et al., 2000). In dogs, gastrointestinal lymphoma showed higher mRNA levels of *Mdr-1* in all cases which is similar to our findings, although there was no mention about histopathological grade and immunophenotype in that study (Culmsee et al., 2004). A recent study reported that canine multicentric lymphomas showed higher mRNA levels of *Mdr-1*; however, T-cell type was associated with lower levels in comparison to B-cell lymphomas that showed overexpression (Zandvliet et al., 2015). This study included only cats with T-cell immunophenotype, therefore cats with B-cell lymphoma should be recruited to compare and determine which immunophenotype shows a higher expression. Otherwise, the evidence suggests that *MDR-1* gene expression in untreated human lymphoma is variable with 10 to 50% of tumours being positive, whereas 100% of our cases were positive (Yuen and Sicik, 1994; Sonneveld, 2000). Further studies are needed to draw conclusions about *Mdr-1* gene expression in different anatomic locations of feline lymphoma, as well as immunophenotype, and if there is a difference in the patterns of gene expression in treated cats compared to untreated, as well as its role as a prognostic marker.

Overexpression of *COX-2* gene in IBD cases from gastrointestinal samples of humans and rats, and colonic biopsies of dogs has been observed in comparison to low levels present in control individuals (Sakamato et al., 2005; Jupp et al., 2007). In dogs, mRNA expression was not statistically different between duodenal samples from control and dogs with IBD but higher *Cox-2* level expression was observed in the latter (Dumusc et al., 2014). This may be similar to our findings that cats with IBD tended to present with higher level of *Cox-2* than HCC group. Untreated human lymphoma may present with high *COX-2* gene expression levels and this overexpression may be associated with cell proliferation and angiogenesis (Ohsawa et al., 2006; Østergaard et al., 2009). This may explain that the LGAL group showed higher *Cox-2* gene expression compared to cats with IBD. Furthermore, *COX-2* upregulation in human lymphomas has been associated with the aggressiveness of the tumour, relapse, poor response to therapy and short overall survival (Hazar et al., 2006; Paydas et al., 2007; Sugita et al., 2007; Ma et al., 2012). This aspect was not evaluated in our study because follow-up was not available. Studies with a larger group of cats with IBD and different grades of AL, and follow-up would be needed to obtain conclusions.

Correlation between COX-2 and P-gp immunoexpression has been reported in hepatocellular cancer cells, breast cancer cells and ovarian cancer cells in human beings (Fantappie et al., 2002; Surowiak et al., 2005; Surowiak et al., 2006). One report about human colorectal cancer described correlations between both genes since blocking

COX-2 downregulated the expression of the *MDR-1* gene (Sui et al., 2001). However, no correlation was shown in our study, similar to a previous study in human Non-Hodgkin's lymphoma (Szczuraszeck et al., 2009). Future studies are needed in cats to confirm the correlation of the studied genes and to determine if COX-2 inhibitor treatment may be beneficial for LGAL.

The study has several limitations. The diagnosis of LGAL was made based on histopathology and immunohistochemistry features; both combined techniques have a sensitivity of 78% and specificity of 99% (Kiupel et al., 2011). Addition of PCR for antigen receptor rearrangements increases sensitivity to 82.6%, but this test was not performed in the present study; thereby, misdiagnosis is possible (Kiupel et al., 2011). Furthermore, only duodenal biopsies were included in this study which it may have influenced the results of the correlations since both diseases may also affect the jejunum and ileum (Jergens, 2012; Lingard et al., 2009; Kiupel et al., 2016). However, the main objective of the study was to describe the gene expression of inflammatory and tumour tissues. Another fact is the small number of sick cats likely affecting the power of the statistical analysis. In addition, the small number of HCC did not allow to be included in the statistical analysis and the results should be considered with caution due to the reduced number of cats with IBD and LGAL. Larger number of cats with IBD and follow-up information would be needed to differentiate them into food-responsive and steroid-responsive groups. Furthermore, follow-up on cats would be necessary to

determine the role of *Mdr-1* and *Cox-2* genes in resistance to treatment and prognosis in cancer as well as IBD.

CONCLUSION

Mdr-1 and *Cox-2* gene expression is higher in cats with LGAL compared to cats with IBD. These results suggest that these genes may be involved in the pathogenesis of IBD and/or LGAL in cats.

5. GENERAL DISCUSSION

IBD affects to middle and old age cats whereas LGAL is reported mainly in older ones although overlap may be present (Norsworthy et al., 2013; Kiupel et al., 2011; Scott et al., 2011). This fact was confirmed by the three presented studies. Sexual predisposition apparently is not observed but some studies have shown higher proportion of sick male cats in concordance to the results reported by the present studies (Guilford et al., 2001; Jergens et al., 2010; Jergens, 2012; Richter, 2003; Barrs and Beatty, 2012a; Norsworthy et al., 2013; Kiupel et al., 2011; Maunder et al., 2012). Domestic shorthair is the breed most commonly affected by chronic enteropathies followed by Siamese, these two were over-represented in our general population in Barcelona and London (Guilford et al., 2001; Jergens et al., 2010; Jergens, 2012; Richter, 2003; Barrs and Beatty, 2012a; Norsworthy et al., 2013; Kiupel et al., 2011; Maunder et al., 2012). It has been suggested that Siamese breed is predisposed to IBD but no studies has confirmed it (Jergens, 2012). In the study I and III, cats with IBD presented a higher body weight (BW) than cats with LGAL, even the BCS was higher in the IBD group in the study performed in UK in agreement with previous reports (Guilford et al., 2001; Jergens et al., 2010; Jergens, 2012; Richter 2003; Barrs and Beatty, 2012a; Norsworthy et al., 2013; Kiupel et al., 2011; Maunder et al., 2012; Freiche et al., 2016). However, unexpectedly, cats with LGAL of the study II showed a higher BW than those with IBD but BCS was not available that correlates better with the body fat and muscle rather than BW (Laflamme, 1997).

Clinical signs described in our studies for cats with IBD and LGAL are similar to previously reported such as weight loss, followed by vomiting, lethargy, inappetence, and diarrhoea (Guilford et al., 2001; Jergens et al., 2010; Jergens, 2012; Richter, 2003; Barrs and Beatty, 2012a; Norsworthy et al., 2013; Kiupel et al., 2011; Maunder et al., 2012; Freiche et al., 2016; Fragkou et al., 2016). These clinical signs were described in detail in the study III but the other ones described the FCEAI that includes vomiting, diarrhoea, anorexia, weight loss and lethargy in the scoring (Jergens et al., 2010).

Clinicopathological alterations found in the study III are also similar to the previously reported in cats with IBD and LGAL though none cats of this latter group presented anaemia that is a common finding (Jergens et al., 2010; Barrs and Beatty, 2012a; Freiche et al., 2016; Fragkou et al., 2016). Otherwise, hypoproteinaemia, hypoalbuminaemia and hypocobalanaemia were more frequent in cats with LGAL as expected and previously described (Barrs and Beatty, 2012; Kiselow et al., 2008). Some clinicopathological parameters were summarized in the study I and II through the FCEAI because includes ALT/ALP and phosphorus (Jergens et al., 2010).

FCEAI is used for defining disease activity on feline IBD or food-responsive enteritis but scarce information is available (Jergen et al., 2010). In these three studies, FCEAI was used in patients with IBD and LGAL. To the author's knowledge, FCEAI has never been applied in cats with LGAL. Scores obtained for IBD group are similar to the results showed by Jergens et al. (2010). On the other hand, cats with LGAL of the

three present studies showed a significative higher score than IBD. This increased score is likely due LGAL is a more severe disease in comparison with IBD, but overlap was observed between both groups. Further prospective studies with a larger number of cats are needed to conclude whether FCEAI may help to differentiate between IBD and LGAL.

Concurrent comorbidities may be present as was reported in the study III. A recent report described that cholangitis is more commonly associated with IBD than pancreatitis followed by triaditis (Fragkou et al., 2016). Regardless, in our study the fPLI was increased in 50% of cats suffering IBD being highly suggested of pancreatitis compared to only 2 cats with cholangitis confirmed by histology and none presented triaditis, however pancreatic biopsies were not obtained to draw conclusions (Xenouli**s,** 2015). Other comorbidities diseases such as those described in the study III have been commonly reported in adult and elderly cats (Fragkou et al., 2016; Puig et al., 2015; Caney, 2009; Foster et al., 2004). Comorbidities were not determinate in the studies performed in Spain because it was one of the criteria for exclusion of both reports and was not the objective of them.

Lymphoplasmocytic enteritis (LPE) has been the most common type of IBD diagnosed in previous reports (Jergens, 2012; Norsworthy et al., 2013; Briscoe et al. 2011; Daniaux et al., 2014). The study I and III included only LPE because P-gp and

Mdr-1 are presented mainly in lymphocytes. Otherwise, eosinophilic enteritis was the second more common inflammation observed in the study II and this type of inflammation was included in this study because eosinophils may induce more severe inflammation.

T cell phenotype is the most common type of LGAL thus only this was included in the three studies (Barrs and Beatty, 2012; Norsworthy et al., 2013; Kiupel et al., 2011; Miyoshi et al., 2002; Moore et al., 2012; Lingard et al., 2009). Furthermore, there is controversy about B cell phenotype is a tumour that emerges in the small intestine due to its origin is from stomach, colon and ileo-colic valve (Moore et al., 2012). Previous reports indicate the most common localizations of LGAL are jejunum and ileum that was confirmed in the studies performed in Spain (Barrs and Beatty 2012). Only duodenal biopsies were obtained by upper GI endoscopy in the study III and this technique allows evaluating till proximal jejunum, and none samples were obtained from the ileum by lower endoscopy. Therefore, misdiagnosis could not be ruled out in this study because LGAL may be present only in the ileum but this should not influence the results of gene expressions over inflammation or tumour from the biopsy obtained (Scott et al. 2011).

According to the WSAVA template, mild or moderate enteritis are more frequently described in accordance to our findings in the IBD group (Briscoe et al., 2011; Maunder et al., 2012; Daniaux et al., 2014; Marsilio et al., 2011). Unexpectedly,

cats of the healthy control group showed mild inflammation without clinical signs and not morphological change according to WSAVA template in the study I and II; this finding is supported by previous studies where cats without gastrointestinal signs had mild inflammation (Nguyen et al., 2006; Fragkou et al., 2016). In the study III, WSAVA template was not used due to lack of consensus among pathologists about this scoring despite the pictorial template and simplification (Willard et al., 2011). Therefore, it was made a scoring according to severity of IBD in all cats.

A previous study in cats found correlation between WSAVA template and FCEAI according to the study I (Jergens et al., 2010). On the other hand, study II results agreed with the studies reported in dogs (Procoli et al., 2013; Allenspach et al., 2007). This discrepancy between study I and II likely is due to cats with eosinophilic enteritis were not included in the study I. It is thought that eosinophilic enteritis presents with more severe clinical signs that may be influence over the FCEAI (Jergens,, 2012). Furthermore, study I included 9 cats with LPE and study 8 with LPE and 3 with eosinophilic enteritis.

Maunder et al. (2012) showed severe histological changes in LGAL applying modified WSAVA, but herein in the study I and II was found moderate ones. These differences may be due to we included a higher number of patients compared to that previous study. Significant difference was observed between LGAL and IBD group in

our studies as reported by Maunder et al. (2012), however further studies are needed to determinate whether this modified scoring might help to differentiate between chronic enteropathies.

To our knowledge, the study I is the first report about P-gp expression in the intestinal epithelium and lamina propria (LP) of cats with IBD and LGAL. Epithelial P-gp immunoexpression in small and large intestine is normal in cats and humans, but in dogs is only present in colon (Van Der Heyden et al., 2009; Van Der Heyden et al., 2011; Cordon-Cardo et al., 1990). Additionally, in canine small intestine the epithelial expression of P-gp is induced by an inflammatory process (Van Der Heyden et al., 2011). P-gp expression at the LP is absent in healthy humans, cats and dogs (Van Der Heyden et al., 2009; Cordon-Cardo et al., 1990; Allenspach et al., 2006). In our study, cats from the control group presented normal epithelial P-gp immunolabelling (Van Der Heyden et al., 2009). Regarding *Mdr-1* gene expression in the study III, the healthy control cats (HCC) showed mild mRNA levels in the duodenum but its origin is indeterminate because separated evaluation of intestinal epithelium and LP should be performed to localize where this gene is encoded. Furthermore, a study evaluating all feline intestinal segments is needed to determine the *Mdr-1* gene expression in all GI tract because human beings present intermediate *MDR-1* mRNA expression in jejunum and colon but duodenum and ileum have not been evaluated whereas in mice maximal expression is in ileum and colon, and gene levels gradually decline proximally toward

stomach (Fojo et al., 1987; Nooter and Herweijer, 1991; Croop et al., 1989; Chianale et al., 1997). Therefore, probably different expressions between species should be considered.

In the study I and III, LGAL group showed higher total P-gp immunolabelling and *Mdr-1* gene expression compared to the cats with IBD and HCC. P-gp expression was marked in the LP in cats with LGAL but *Mdr-1* could not be possible to determine its origin because was examined the mucosa. Epithelial P-gp expression is mainly discontinuous or abnormal in the lymphoma group likely due to the epitheliotropism of this tumour. Otherwise, cats with IBD could showed several expressions of P-gp in the LP but when only moderate one was evaluated, IBD was significantly higher in comparison with LGAL and HCC groups. Regarding *Mdr-1* gene, IBD group showed a higher mRNA levels whereas HCC group tended to show lower levels. These results suggest that P-gp and *Mdr-1* gene may be involved in the pathogenesis of feline IBD and/or LGAL and might be implicated in the need of a more aggressive treatment in the LGAL and prognosis.

COX-2 products might be involved in maintaining the integrity of intestinal mucosa (Kefalakes et al., 2009). Regarding immunoreactivity in healthy feline GI tract, only one study described COX-2 immunoexpression in basal granulated cells of the epithelium using a polyclonal antiprostaglandin H synthetase-2 (COX-2) human C terminus antibody (Satoh et al., 2013). Nonetheless in the study II, immunolabelling was

found in the cytoplasm of the enterocytes in 3 healthy indoor cats and 2 sick. The discordance about localization of the reactivity may be explained by the different anti-reagent used, non-specific staining and aberrant antibody binding, but the antibody used herein was previously reported for cats (Hayes et al., 2006; Newman et al., 2006; Bardagí et al., 2012; Jackson et al., 2000; Paiotti et al., 2007; Singer et al., 1998; Joo et al., 2002; Joo et al., 2003). Finally, not all control cats showed epithelial COX-2 expression as healthy humans since 50 to 80% present COX-2 expression in colon and stomach (Jackson et al., 2000; Paiotti et al., 2007; Romero et al., 2008; Dai et al., 2015). Further studies with larger number of cats are needed to obtain conclusions about normal COX-2 expression in the gastrointestinal tract. Epithelial intensity immunoexpression in IBD and LGAL groups was significant higher in comparison with control group. This higher expression may be due to the increased levels of prostaglandin E_2 though no statistical difference was found between diseased groups (Singer et al., 1998). A hypothesis might be COX-2 increases by a protective mechanism to health the intestinal mucosa as constitutive enzyme or other mechanisms induced by cytokines as inflammatory response. No expression was observed in the LP in cats with IBD neither HCC group, however 3 cats with LGAL presented with COX-2 immunoexpression. Furthermore, cats with LGAL showed higher levels of *Cox-2* gene expression compared to IBD. *COX-2* upregulation has been associated with the aggressiveness of the tumour, relapsed, worst response to therapy and less overall

survival (Hazar et al., 2006; Paydas et al., 2007; Ma et al., 2012; Sugita et al., 2007). This latter could not be determined in our studies because not all cats had available the follow-up and it was not an aim. Therefore, studies with a larger group of cats with IBD, LGAL and intermediate-high grade lymphoma, and follow-up would be needed to obtain conclusions.

Only correlation between P-gp in the LP and FCEAI and histological scores was observed likely because severe clinical signs, severe inflammation and tumour cells infiltration show more marked expression of this protein. This severity may be reflected with higher expression of P-gp in the LP that might be related with the treatment response and prognosis of IBD and LGAL, but further studies are needed to confirm this hypothesis.

These studies have some limitations in common, they included small size of sick cats and they are retrospectives. Even the study III recruited a small number of HCC that was not included in the statistical study, thus the results should be interpreted with caution. In addition, the FCEAI was calculated by record data or owner interview by phone, therefore subjectivity is an uncontrolled variable. Regarding WSAVA template used in study I and II, most of the cats had full-thickness biopsies and different intestinal segments were studied but this scoring has been designed only for stomach, duodenal and colonic endoscopic biopsies but previous canine studies have used in jejunum, ileum and full-thickness biopsies (Day et al., 2008; Procoli et al., 2013;

Casamian-Sorrosal et al., 2010). However, one pathologist evaluated all biopsies thus interobserver variation disappeared (Willard et al., 2002). Cases of triaditis were not included in study I and II, but did in study III, however fPLI was unavailable when many cases were diagnosed and pancreatitis was ruled out only by ultrasound. Moreover, PARR was not performed mainly in the study II where only endoscopy biopsies were used and misdiagnosis could have occurred. However, immunohistochemistry was performed increasing the sensitivity and specificity of histopathological diagnosis (Kiupel et al., 2011).

Further studies are needed with larger number of HCC, cats with IBD, LGAL, and other types of AL, their follow-up would be needed to split up in food-responsive and steroid-responsive enteropathy group and to determine their genetic and immunoexpression. Furthermore, follow-up would be necessary to determine the P-gp, *Mdr-1* and COX-2 involvement in pathogenesis, resistance to treatment and prognosis in feline IBD and AL.

6. CONCLUSIONS

1. Cats with LGAL tend to show a higher FCEAI score than cats with IBD due to lymphoma is a more severe disease but overlap was observed between both diseases.

2. LGAL group shows higher modified WSAVA score in comparison with IBD group because lymphoma produces more severe morphological alterations secondary to the tumour cell infiltration.

3. Sixty percent of cats with IBD present P-gp expression in the LP that might be involved in the pathogenesis of the disease.

4. Most of cats with LGAL present discontinuous or abnormal P-gp immunoexpression in the intestinal epithelium compared to cats with IBD that might be associated with the epitheliotropim and severity of the disease.

5. Most of cats with LGAL show marked immunostaining in the LP compared to cats with IBD that might be associated with the severity of the lymphoma and the need of a more aggressive treatment.

6. Higher expression of P-gp in the LP is associated with more severe histological alterations that might be also implicated with the severity of the disease and resistance to the treatment or more aggressive therapy.

7. Increased COX-2 intensity in the epithelial cells is observed in cats with IBD and LGAL that may be secondary to the inflammatory response or a protective function in the intestinal reparation.

8. No differences are observed regarding COX-2 expression at the LP between all groups but it was presented in only 33% of cats with LGAL thus higher number of patients with lymphoma may be needed to stablish differences and importance of this expression in the pathogenesis and treatment.

9. *Mdr-1* and *Cox-2* gene expression is higher in cats with LGAL compared to cats with IBD that may suggest that these genes may be involved in the pathogenesis of IBD or LGAL in cats.

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APPENDIX 1

FELINE CHRONIC ENTEROPATHIES STUDY

Selection criteria

-Adult Cats (> 1 year-old)

-History of gastrointestinal (GI) signs: chronic diarrhea with or without vomiting of at least 2 to 3 weeks duration (Fill the FCEAI sheet)

Standard diagnostic tests (minimum data base)

CBC, Biochemical analysis, urinalysis, fecal examinations for parasites (direct smear and zinc sulfate flotation test), PCR or culture *Tritrichomonas foetus* (≤ 3 years old), abdominal ultrasound, total T4 (≥ 5 years old).

Pathologic markers (keep frozen serum to measure these parameters in future)

Albumin, phosphorous, cobalamin, folate, fPLI, fTLI, ALT/ALP

-Rule out intestinal lymphoma and other extra gastrointestinal diseases

-Upper/Lower Endoscopy: ≥ 5 adequate biopsies from each gastrointestinal area

-None of the cats should have been treated with antibiotics, corticosteroids, or antacids in the 2 weeks before remitting.

Instructions

Pages 1-3 should be filled for the clinician

Pages 4- 8 should be filled for the endoscopist

Pages 9- 13 should be filled for the pathologist/clinician

Page 14 should be filled for the clinician

References

WSAVA gastrointestinal standard group

Jergens AE, Crandell JM, Evans R et al. A clinical index for disease activity in cats with chronic enteropathy. J Vet Intern Med, 2010; 24:1027-1033.

Name: **ID:** **Date:**

Patient Information

Age: _____ Breed: _____ Female □ Neutered □

Kg: _____ Body Condition Score: _____ Male □ Non-neutered □

Environmental History

Indoor □ Outdoor□ Both□

Exposure to parasites: _____

Contact with uncontrolled animals: _____

Past Medical History

FeLV/FIV status: _____

Vaccination status: _____

Worming status: _____

Previous abdominal surgery: _____

Previous excision of cutaneous mast cell tumor: _____

Previous diseases: Gingivostomatitis □ Skin allergies □ Others□

_____

_____

_____

FELINE CHRONIC ENTERITIS ACTIVITY INDEX (FCEAI)

Current history: _____

_____

_____

GI signs	0	1	2	3	SUBTOTAL
Attitude/activity	Normal	Slightly decreased	Moderately decreased	Severely decreased	
Appetite	Normal	Slightly decreased	Moderately decreased	Severely decreased	
Vomiting	None	Mild (1X/wk)	Moderate (2-3X/wk)	Severe (>3X/wk)	
Diarrhea	None	Slightly soft feces or blood/mucus or slightly increased (2-3X/d)	Very soft feces or moderately increased (4-5X/d)	Watery diarrhea or severely increased (>5X/d)	
Weight loss	None	Mild (<5%)	Moderate (5-10%)	Severe (>10%)	

Diagnostic Tests (Mark the □ with an X and write the abnormal values, N=normal):

□ FeLV/FIV test _____

□ CBC _____

□ Biochemistry profile _____

□ Urinalysis _____

□ Fecal examination _____

□ Fecal bacteria culture _____

□ *Tritrichomonas foetus* _____

□ TT4 _____

□ Ultrasound _____

□ Rx _____

Pathologics markers

□ Albumin _____

□ Total proteins _____

□ ALT/ALP _____

□ Phosphorous _____

□ Cobalamin _____

□ Folate _____

□ *f*PLI _____

□ *f*TLI _____

Treatment pre-endoscopy

□ Antiparasitic medicament:

_____

□ Diet, Which one? _____ Good response: □YES □NO

_____

□ Metronidazole □ Tylosin □ Marbofloxacin Good response: □YES □NO

_____

□ Immunosuppressive Drugs, Which one? _____

_____

_____

□ **Endoscopy/biopsies** (≥ 5 biopsies for each segment)

□ **Laparatomy or Laparoscopy/Full thickness biopsy**

ENDOSCOPIC EXAMINATION REPORT: UPPER GI ENDOSCOPY

Date of procedure: ..... **Case Number:** .....

Patient and client information

PROCEDURE(S): _____

Indication(s) for procedure: _____

Endoscope(s) used: _____

Forceps/retrieval device(s) used: _____

PROBLEMS/COMPLICATIONS: None □

Perforation □ Excessive bleeding □ Anesthetic complications □

Excessive time □ Other □

Comments: _____

□ Unable to complete full examination: why? _____

□ Unable to obtain adequate biopsies: why? _____

□ Unable to retrieve foreign object: why? _____

□ Visualization obscured why? _____

SAMPLING: Biopsy □ Brush cytology □ Washing □ Aspiration □ Foreign body retrieved □

DOCUMENTATION: Video □ Photographs □

ESOPHAGUS Normal □ Foreign body □ Mass □ Stricture □ Hiatal hernia □

Lesion	Code	Comments (include location)
Hyperemia/vascularity		
Discoloration		
Friability		
Hemorrhage		
Erosion/ulcer		
Contents (mucus/bile/food)		
Dilation		
Gastroesophageal sphincter		
Other		

Code: Normal = 0 Mild = 1 Moderate = 2 Severe = 3

STOMACH Normal □ Foreign body □ Mass □ Polyp(s) □ Parasite(s) □

Site(s) of lesions: Fundus □ Body □ Incisura □ Antrum □ Pylorus □

Site(s) of biopsies: Fundus □ Body □ Incisura □ Antrum □ Pylorus □

Lesion	Code	Comments (include location)
Can't ínflate lumen		
Hyperemia/vascularity		
Edema		
Discoloration		
Friability		
Hemorrhage		
Erosion/ulcer		
Contents (mucus/bile/food)		
Gastroesophageal sphincter		
Passing scope through pylorus		
Other		

Code: Normal = 0 Mild = 1 Moderate = 2 Severe = 3

DUODENUM/JEJUNUM Normal □ Foreign body □ Mass □ Polyp □ Parasite(s) □

How far was the tip of the scope advanced?_____

Was/were the papilla(e) seen? Yes □ (which? _____) No □

Lesion	Code	Comments (include location)
Can't ínflate lumen		
Hyperemia/vascularity		
Edema		
Discoloration		
Friability		
Hemorrhage		
Erosion/ulcer		
Contents (mucus/bile/food)		
Gastroesophageal sphincter		
Passing scope through pylorus		
Other		

Code: Normal = 0 Mild = 1 Moderate = 2 Severe = 3
Comments and Recommendations: _____
_____
_____
_____

ENDOSCOPIC EXAMINATION REPORT: LOWER GI ENDOSCOPY

Date of procedure: ..... **Case Number:** .....

Patient and client information

PROCEDURE(S): _____

Indication(s) for procedure: _____

Endoscope(s) used: _____

Forceps used: _____

Method of preparing colon: _____

PROBLEMS/COMPLICATIONS: None □ Colonic preparation inadequate □

Perforation □ Excessive bleeding □ Anesthetic complications □ Excessive time □ Other □

Comments: _____

□ Unable to complete full examination: why?_____

SAMPLING: Biopsy □ Brush cytology □ Washing □ Aspiration □

□ Unable to obtain adequate biopsies: why? _____

□ Visualization obscured why? _____

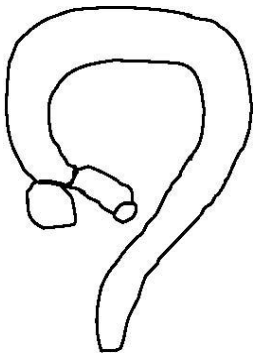
DOCUMENTATION: Video □ Photographs □

COLON Normal □ Foreign body □ Parasite(s) □ Mass □ Polyp □

Visualized: ileo-colic valve □ ceco-colic valve (dog) □ cecum (cat) □

If did not see ileo-colic valve area, how far was the scope advanced? _____

_____

Lesion	Code	Comments (include location)	
Hyperemia/vascularity			
Discoloration			
Friability /Hemorrhage			
Erosion/ulcer			
Intussusception			
Stricture			
Artifact			
Other			

Code: Normal = 0 Mild = 1 Moderate = 2 Severe = 3

ILEUM NOT EXAMINED □

□ Tried to pass scope through ileocolic valve: Successful □ Unsuccessful □

□ Tried to biopsy the ileum: Successful □ Unsuccessful □

Biopsies taken by: Direct visualization □ Blindly passing forceps through ileocolic valve □

Normal □ Foreign body □ Parasite(s) □ Mass □

Lesion	Code	Comments (include location)
Can't inflate lumen		
Hyperemia/vascularity		
Edema		
Discoloration		
Friability/Hemorrhage		

Erosion/ulcer		
Lacteal dilatation		
Texture of mucosa		
Mass		
Other		

Code: Normal = 0 Mild = 1 Moderate = 2 Severe = 3

CECUM NOT EXAMINED □

□ Tried to intubate the cecum (dogs): Successful □ Unsuccessful □

Normal □ Foreign body □ Parasite(s) □ Mass □

Lesion	**Code**	**Comments (include location)**
Can't inflate lumen		
Hyperemia/vascularity		
Edema		
Discoloration		
Friability/Hemorrhage		
Texture		
Erosion/ulcer		
Other		

Code: Normal = 0 Mild = 1 Moderate = 2 Severe = 3

Comments and Recommendations: _____

_____

_____

Endoscopist signature _____

STANDARD FORM FOR ASSESSMENT OF THE GASTRIC BODY OR ANTRAL MUCOSA

Pathologist_____ Case number_____

Number of pieces of gastric tissue on slide_____

Tissue present

☐Inadequate ☐Too superficial ☐Adequate depth

Number of tissues abnormal_____

MORPHOLOGICAL FEATURES

	Normal	Mild	Moderate	Marked
Surface epithelial injury	☐	☐	☐	☐
Gastric pit epithelial injury	☐	☐	☐	☐
Fibrosis/glandular nesting/ mucosal atrophy	☐	☐	☐	☐

INFLAMMATION

Intraepithelial lymphocytes	☐	☐	☐	☐
Lamina propria lymphocytes and plasma cells	☐	☐	☐	☐
Lamina propria eosinophils	☐	☐	☐	☐
Lamina propria neutrophils	☐	☐	☐	☐
Other inflammatory cells	☐	☐	☐	☐
Gastric lymphofollicular hyperplasia	☐	☐	☐	☐

FINAL DIAGNOSIS

Normal tissue ☐

Lymphoplasmacytic inflammatory ☐

Eosinophilic inflammatory ☐

Neutrophilic inflammatory ☐

Mucosal atrophy/fibrosis (non-inflammatory) ☐

Other ☐

OTHER COMMENTS

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	Marked
Villous stunting	□	□	□	□
Epithelial injury	□	□	□	□
Crypt distension	□	□	□	□
Lacteal dilation	□	□	□	□
Mucosal fibrosis	□	□	□	□

INFLAMMATION

Intraepithelial lymphocytes	□	□	□	□
Lamina propria lymphocytes and plasma cells	□	□	□	□
Lamina propria eosinophils	□	□	□	□
Lamina propria neutrophils	□	□	□	□
Other	□	□	□	□

FINAL DIAGNOSIS

Normal tissue □

Lymphoplasmacytic inflammatory □

Eosinophilic inflammatory ☐

Neutrophilic inflammatory ☐

Mucosal atrophy/fibrosis (non-inflammatory) ☐

Other ☐

OTHER COMMENTS

STANDARD FORM FOR ASSESSMENT OF JEJUNAL 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	Marked
Villous stunting	☐	☐	☐	☐
Epithelial injury	☐	☐	☐	☐
Crypt distension	☐	☐	☐	☐
Lacteal dilation	☐	☐	☐	☐
Mucosal fibrosis	☐	☐	☐	☐

INFLAMMATION

Intraepithelial lymphocytes	☐	☐	☐	☐
Lamina propria lymphocytes and plasma cells	☐	☐	☐	☐
Lamina propria eosinophils	☐	☐	☐	☐
Lamina propria neutrophils	☐	☐	☐	☐
Other	☐	☐	☐	☐

FINAL DIAGNOSIS

Normal tissue ☐

Lymphoplasmacytic inflammatory ☐

Eosinophilic inflammatory ☐

Neutrophilic inflammatory ☐

Mucosal atrophy/fibrosis (non-inflammatory) ☐

Other ☐

STANDARD FORM FOR ASSESSMENT OF ILEAL 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	Marked
Villous stunting	☐	☐	☐	☐
Epithelial injury	☐	☐	☐	☐
Crypt distension	☐	☐	☐	☐
Lacteal dilation	☐	☐	☐	☐
Mucosal fibrosis	☐	☐	☐	☐

INFLAMMATION

Intraepithelial lymphocytes	☐	☐	☐	☐
Lamina propria lymphocytes and plasma cells	☐	☐	☐	☐
Lamina propria eosinophils	☐	☐	☐	☐
Lamina propria neutrophils	☐	☐	☐	☐
Other	☐	☐	☐	☐

FINAL DIAGNOSIS

Normal tissue ☐

Lymphoplasmacytic inflammatory ☐

Eosinophilic inflammatory ☐

Neutrophilic inflammatory ☐

Mucosal atrophy/fibrosis (non-inflammatory) ☐

Other ☐

OTHER COMMENTS

STANDARD FORM FOR ASSESSMENT OF COLONIC 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	Marked
Surface epithelial injury	☐	☐	☐	☐
Crypt hyperplasia	☐	☐	☐	☐
Crypt dilatation/distortion	☐	☐	☐	☐
Fibrosis/atrophy	☐	☐	☐	☐

INFLAMMATION

Lamina propria lymphocytes and plasma cells	☐	☐	☐	☐
Lamina propria eosinophils	☐	☐	☐	☐

Lamina propria neutrophils ☐ ☐ ☐ ☐

Lamina propria macrophages ☐ ☐ ☐ ☐

FINAL DIAGNOSIS

Normal colon ☐

Lymphoplasmacytic inflammatory ☐

Eosinophilic inflammatory ☐

Neutrophilic inflammatory ☐

Lymphangiectasia ☐

Mucosal atrophy/fibrosis (non-inflammatory) ☐

Other ☐

OTHER COMMENTS

FINAL CLINICAL DIAGNOSIS: _____
_____

Treatment

□ Diet, Which one? _____

□ Antibiotic, Which one? _____

□ Immunosuppressive Drugs, Which one? _____

_____

_____

Follow up (21-28 days later)

Date: _____

Clinical evolution: _____

_____

_____

FCEAI

GI signs	0	1	2	3	SUBTOTAL
Attitude/activity	Normal	Slightly decreased	Moderately decreased	Severely decreased	
Appetite	Normal	Slightly decreased	Moderately decreased	Severely decreased	
Vomiting	None	Mild (1X/wk)	Moderate (2-3X/wk)	Severe (>3X/wk)	
Diarrhea	None	Slightly soft feces or blood/mucus or slightly increased (2-3X/d)	Very soft feces or moderately increased (4-5X/d)	Watery diarrhea or severely increased (>5X/d)	
Weight loss	None	Mild (<5%)	Moderate (5-10%)	Severe (>10%)	

Diagnostic tests repeated and values: _____

_____

_____

_____

New treatment?

□ No, __

_____

_____

□ Yes □ New elimination diet. Which one? _____

□ New antibiotic. Which one? _____

□ New immunosuppressive drug. Which one? _____

Others:_____

_____

_____

APPENDIX 2

List of Abbreviations

IBD Inflammatory bowel disease

GI Gastrointestinal

CE Chronic enteropathy

WSAVA World Small Animal Veterinary Association

AL Alimentary lymphoma

LGAL Low grade alimentary lymphoma

IGAL Intermediate grade alimentary lymphoma

HGAL High grade alimentary lymphoma

PCR Polymerase chain reaction

P-gp Permeability glycoprotein/P-glycoprotein

MDR-1 Multidrug resistance 1

COX-2 Cyclooxygenase 2

mRNA Messenger ribonucleic acid

NSAID Non-steroidal anti-inflammatory drug

TLR Toll-like receptor

NOD nucleotide-binding oligomerization

IL Interleukin

MHC Major histocompatibility complex

FeLV Feline leukemia virus

DNA Deoxyribonucleic acid

FIV Feline immunodeficiency virus

FRE Food responsive enteritis

FCEAI Feline chronic enteropathy activity index

LPE Lymphoplasmacytic enteritis

NCIWF National Cancer Institute Working Formulation

REAL Revised European-American Lymphoma

WHO World Health Organisation

IHC Immunohistochemistry

IELs Intraepithelial lymphocytes

PARR PCR for antigen receptor rearrangement

TCRG T cell receptor gamma

IgH Immunoglobulin heavy chain

LP Lamina propria

HCC Healthy female control cats

SC Sick cats

UAB Universitat Autònoma de Barcelona

Hpf High power field

FTB Full thickness biopsy

GC Glucocorticoids

DSH Domestic shorthair