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Evaluation of serum and fecal parameters in cats with low-grade intestinal T-cell lymphoma (LGITCL)

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ABSTRACT

Lymphoma is the most common neoplasia in the intestine of cats. According to ACVIM consensus statement, low-grade intestinal T-cell lymphoma (LGITCL) represents a monomorphic infiltration of the lamina propria or epithelium or both of cats with small, mature, neoplastic (clonal) T lymphocytes. Despite the importance as contributing factors of inheritance and environment in the pathogenesis of LGITCL, the chronic inflammatory status plays a fundamental role. The aim of the present study was to investigate possible diagnostic and prognostic parameters in LGITCL. Selected fecal bacteria and serum biomarkers (serum amyloid A [SAA]; citrulline; total protein; amylase; lipase; DGGR lipase; cholesterol; lipopolysaccharides [LPS], and zonulin) were evaluated in 12 cats (lymphoma group – LG) with histologically diagnosed low-grade intestinal T- cell lymphoma (LGITCL) and then compared with a control group consisting of 12 clinically healthy cats (CG). The evaluation of fecal bacterial population showed the significant decrease of *Faecalibacterium* spp. ($P = 0,045$) and *Clostridium hiranonis* ($P = 0,0433$) and a significant increase in *E. coli* ($P = 0,045$), *Streptococcus* spp. ($P = 0,0003$) and *Turicibacter* spp. ($P = 0,0056$) in the lymphoma group. Serology showed a significant decrease of total proteins ($P = 0,092$), amylase ($P = 0,092$) and cholesterol ($P = 0,0112$) in LG group compared to controls. The present results allowed the authors to state that in cats LGITCL is possibly associated to a change in the gastrointestinal environment and to a condition of protein losing enteropathy. Further studies with a larger cohort of patients are needed to confirm the present results and to point out other possible changes.

1. Introduction

Intestinal lymphoma is among the most frequent kinds of neoplasm in cats, followed by adenocarcinoma and mast cell tumor (Barrs and Beatty, 2012), and it is classified according to anatomical site of occurrence, histology, immunophenotype and nature of infiltration. Many denominations have been proposed to characterize the neoplastic infiltrative state of the gastrointestinal tract of cats, characterized by a population of low-grade proliferating T lymphocytes, including "small cell lymphoma" (SCL), "low-grade lymphoma" (LGL), alimentary lymphoma (AL), lymphosarcoma, enteropathy-associated T-cell lymphoma (EATL), epitheliotropic monomorphic intestinal T-cell lymphoma (MEITL), and low-grade intestinal T-cell lymphoma (LGITL). Thanks to the work of the ACVIM consensus statement panel guidelines (Marsilio et al., 2023) all these different denominations have been traced back to the definition of low-grade intestinal T-cell lymphoma (LGITCL) for lesions in the gastrointestinal tract of cats with clinical signs of chronic enteritis, characterized histologically by a monomorphic infiltration of the lamina propria or epithelium or both with small, mature, neoplastic (clonal) T lymphocytes. In the diagnostic algorithm of LGITCL to date no specific markers of pathology are recognized and the golden standard for diagnosis still remains biopsy sampling, associated with IHC examination and PARR evaluation. The 12 cats selected in the present study (lymphoma group – LG) showed a LGITCL consisting in a typical intestinal T-cell lymphoma with a low degree of invasiveness, which has a longer course, a less aggressive progression and a symptomatology mainly characterized by slow weight loss, in some cases diarrhea, vomiting and anorexia.

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Several risk factors play a role in the development of this pathology, starting from genetic (Kieslinger et al., 2021; Freiche et al., 2021a), and including environmental (Bertone et al., 2002), infectious and inflammatory factors. Chronic inflammation leading to the alteration of the mechanisms that regulate cell replication and increasing oxidative stress and damage to cellular DNA, represents the major risk factor in the neoplastic transformation of intestinal lymphocyte populations (Kiupel et al., 2011; Moore et al., 2012; Moore et al., 2005).

The intestinal microbiota consists of bacteria, archaea, fungi, viruses and protozoa that occupy the gastrointestinal tract (Deng and Swanson, 2015; Suchodolski, 2016). Main roles played by the gastrointestinal (GI) well balanced microbiota (in a condition of eubiosis) are the protection against opportunistic pathogens, an "appropriate" immune stimulation of the GI lymphatic tissue (determining a correct "*oral tolerance*") and the production of specific beneficial metabolites (e.g., *C. hiranonis* and secondary bile acids). Therefore, a condition of dysbiosis, i.e., an alteration of the composition and/or richness of the microbiota, influences the health status of the host. During inflammatory diseases, a state of dysbiosis is constantly observed, even if it is not yet clear whether the dysbiosis precedes the gastrointestinal disorders or vice versa; however, it is certain that a state of inflammation can determine a dysbiosis which aggravates the triggering inflammatory condition (Suchodolski, 2016). Contrary to what has been developed in human medicine, models of dysbiosis linked to a specific gastrointestinal alteration have not yet been proposed in veterinary medicine, but a dysbiosis index (DI) has been validated. The DI is calculated by applying a mathematical and statistical model that "measures" the abundance of selected bacteria and provide the information whether the GI microbiome is eubiotic or not (Alshawaqfeh et al., 2017). Similarly, to what happens during chronic inflammation, also in the determinism of intestinal neoplasia it is hypothesized that the basis of the alteration of the cell cycle is the production of carcinogenic metabolites and toxins associated to an increased inflammatory response by the altered microbiota (García-Castillo et al., 2016). Consequently, a persistent inflammatory stimulus can induce the onset of a neoplastic condition, especially of the lymphoid lineage as it is constantly recalled to the sites of inflammation. The relationship between dysbiosis and cancer is to be considered bivalent as dysbiosis can determine cancer, and vice versa cancer can alter the environment by inducing a condition of dysbiosis which stimulates carcinogenesis (Zou et al., 2018). The aim of the present study was to investigate possible diagnostic and prognostic parameters in cats with low-grade intestinal t-cell lymphoma. Numerous serum markers are able to provide information on the inflammatory condition and on the functionality and integrity of the intestinal barrier (Cerón et al., 2005). Serum amyloid A (SAA), a major acute phase protein in cats, allows us to evaluate the inflammatory condition and varies in a very rapid but unspecific way (Cerón et al., 2005). Citrulline, produced by enterocytes, represents a specific marker of intestinal function and mass (Barzał et al., 2014). Total proteins are mainly made up of albumin, an acute phase protein which tends to decrease during inflammation; hypoalbuminemia may also be related to protein-losing enteropathies (Willard et al., 2004). Amylase and lipase are non-specific markers of pancreatic disease. Amylase is filtered by renal tubules and resorbed by tubular epithelium, small amounts are taken up by Kupffer cells in the liver, finally it is produced also by the epithelium of the small intestine; therefore, its altered concentration may be associated to enteropathies (Willard et al., 2004). Lipase and DGGR lipase (novel technique using 1,2-o-dilauryl-rac-glycerol-3-glutaric [6-methylresorufin] ester or DGGR) increase in serum in non-pancreatic disorders such as renal failure, liver, intestinal diseases and in particular lymphoma (Steiner, 2003; Graca et al., 2005). Cholesterol is mainly produced by hepatocytes and, extrahepatically, also by the cells of the intestinal mucosa; its decrease can thus be determined by a protein-losing enteropathy (Willard et al., 2004; Le Roy et al., 2019). In addition, during lymphoplasmacytic enteritis (LPE) as well as in severe small intestinal epithelium infiltration/damage promoted by LGITCL, a reduction of the influx of cholesterol across the apical brush border membrane of the enterocyte may be produced (Wang, 2003). Zonulin is an intracellular protein associated with *zonula-occludens* which, by modulating the intercellular tight junctions, regulates the permeability of the intestinal barrier and is released into the circulation or into the intestinal lumen following inflammation or enteric pathologies that break the intercellular bonds. An increase in plasma or feces may therefore indicate damage to the enterocyte barrier and can consequently be evaluated as a marker of intestinal integrity. In particular, high levels of serum Zonulin are suggestive of increased intestinal permeability and are found in patients presenting with chronic inflammatory, immune-mediated or neoplastic intestinal disorders (Sturgeon and Fasano, 2016; Ficek et al., 2017). Bacterial lipopolysaccharide (LPS), an endotoxin constituting the lipid component of the Gram- bacterial wall, is strongly pro-inflammatory and increases in the plasma following an altered permeability of the intestinal barrier responsible for an increase in bacterial translocation (Hasegawa et al., 2015; Sturgeon and Fasano, 2016). The aim of the present study was to investigate possible diagnostic and prognostic parameters in cats with low-grade intestinal t-cell lymphoma. Selected fecal bacteria and the above-mentioned serum markers were investigated in 12 cats presenting LGITCL, and compared with 12 healthy controls, to evaluate their possible role as biomarkers with diagnostic and prognostic purposes and showing a possible association to a change in the gastrointestinal environment and to a condition of protein losing enteropathy with LGITCL.

2. Materials and methods

2.1. Patients enrolled in the study

2.1.1. Lymphoma group

Twelve cats (5 males and 7 females) diagnosed with LGITCL by histopathology, immunohistochemistry and PCR for antigen receptor rearrangement (PARR), mean age of 9.7 years, were include in the study (LG). All patients underwent abdominal ultrasonography and gastrointestinal endoscopy (GI), plus biopsy samplings. Samples resulted histologically positive were subsequently subjected to immunohistochemistry staining (for CD3, expressed by T cells, and CD20 expressed by B cells) for immunophenotyping the process, and PCR for antigen receptor rearrangement. Patients enrolled in LG were variably presenting diarrhea from at least one month, with a concomitant reduction of 1–2 points of the BCS. All cats were serologically negative for FIV, FeLV and FIP and no steroids had been administered in the month prior to biopsy and serum samplings. Direct (wet mount) and indirect (flotation) examination of feces for endoparasites were also performed. Copro-microscopic and *Giardia* evaluations were also performed, resulting negative, as inclusion criteria in the study.

2.1.2. Control group

The control group (CG) consisted of 12 healthy cats (8 male and 4 females; mean age of 8.5 years), living in home environments and free of gastrointestinal signs for at least four months, clinically and laboratoristically evaluated for annual planned health screenings. All cats were judged to be healthy based on normal results on physical examination, complete blood count, serum biochemistry, urinalysis, repeated fecal examinations (fecal coprological evaluation performed on 3 consecutive days), and negative serology for FIV, FeLV and FIP. In all 12 cats, physical examination was performed, and the body condition score was assessed using a previously established a nine-point condition scoring system (Bjornvad CR, et al. Evaluation of a nine-point body condition scoring system in physically inactive pet cats. Am. J. Vet. Res. 2011;72:433–437. doi: https://doi.org/10.2460/ajvr.72.4.433.). No sedation was required for clinical evaluation, or blood collection in this group of animals. Blood was collected from a peripheral vein or the jugular vein and the following tests were performed: complete blood count, serum chemistry profile, total protein electrophoresis, total T4,

serum amylase, lipase and DGGR lipase, cholesterol, and feline trypsin-like immunoreactivity (fTLI). Cats with gastrointestinal signs (weight loss, hyporexia, vomiting > 2×/month, diarrhea) within 6 months prior to enrollment were excluded. In addition, cats with systemic diseases, chronic illnesses or clinically significant laboratory abnormalities were excluded from the study.

Finally, cats that had received any antibiotics, antacids, anti-inflammatory drugs, or corticosteroids within the past 4 months prior to sampling, were excluded. No treatments (i.e., antibiotics, antacids, anti-inflammatory drugs or corticosteroids) had been administered within the past 4 months prior to sampling. A BCS of 3/5 (normal) characterized all cats.

All cats enrolled in this study were fed various commercial pet foods or home-prepared diets.

Imaging findings, endoscopic histopathologic examinations and feline CE activity index (FCEAI) (Jergens et al., 2010) were recorded.

2.1.3. Endoscopical examination

For endoscopical examination, food was withheld from cats for at least 12 h before each procedure; Medetomidine (0.03 mg/kg [0.014 mg/lb]) was administered IM as premedication. The doses were chosen based on those commonly used in cats at the University of Camerino Teaching Hospital for Animals. Twenty minutes after administration of premedicant, the degree of sedation was evaluated, then general anesthesia was induced with ketamine (10 mg/kg [4.5 mg/lb], IM). Cats lay on a circulating warm-water blanket throughout the entire anesthetic period. Ten minutes after ketamine administration, orotracheal intubation was attempted; in cats that were insufficiently anesthetized to allow orotracheal intubation, isoflurane was provided via a face mask until of a pediatric circle-rebreathing system, and cats were allowed to breathe spontaneously. End-tidal carbon dioxide partial pressure and isoflurane concentration were monitored and recorded every 5 min. When end-tidal isoflurane concentration was stable at 1.4 % for 15 min, endoscopy was initiated. The endoscopy was performed by use of a gastrointestinal videoscope with an 8.5-mm insertion tube diameter and 103-cm working length. An expert endoscopist (GP) performed the exam.

2.1.4. Informed consent and questionnaire

The enrolled cats and their owners received written information on methods, according to previous studies (Lemaire, 2006), and all owners gave their written informed consent to participate in the study. In addition, for the 12 health cats constituting the control group, additional questions were posed to the owners, regarding on cat's general and gastrointestinal health. The questionnaire covered different areas, as well as attitude/activity, appetite, drinking, urination, chronic illnesses, weight loss, vomiting, diarrhea, and treatment with antibiotics, antacids, anti-inflammatory drugs, or steroids. The collection and analysis of intestinal biopsies obtained endoscopically from cats included in the study were performed for clinical purposes, using routine techniques, respecting National Laws on Studies Involving Animals.

2.1.5. Fecal Bacteria

All fecal samples of both groups were collected within 3 h after natural evacuation and immediately frozen at −80 °C until the examination. Total fecal DNA was extracted using the QIAamp PowerFecal Pro DNA Kit (QIAGEN) and an automatic extraction system (Thermo KingFisher Flex Magnetic Particle Purification 96 PCR Isolation System), according to the manufacturers' instructions. The qPCR assays were performed as previously reported (AlShawaqfeh et al., 2017; Blake et al., 2020). Briefly, the DNA concentration of the extract was measured by a spectrophotometer (NanoDrop 1000; Thermo Scientific) and normalized to 5 ng/μl. A mixture of 2 μl normalized DNA extract (5 ng/μl), 5 μl SsoFast EvaGreen supermix (Bio-Rad Laboratories), 0.4 μl forward primer (400 nM), 0.4 μl reverse primer (400 nM) and 2.2 μl DNA-free water was used for qPCR assays using a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad Laboratories). The protocol for the thermal cycler was described elsewhere (Sung et al., 2022), and the qPCR results were analyzed by the Bio-Rad CFX Maestro 1.1 software (Bio-Rad Laboratories). The 10 targeted bacterial groups, the primer sets and their specific annealing temperatures are previously described (Sung et al., 2022), as well as all tests to verify the specificity of the PCR primers. The PCR product was extracted from the gel using the QIAquick Gel Extraction Kit (QIAGEN) and ligated to pCR 4-TOPO vector (Invitrogen) and transformed into competent DH5aTM-T1R *E coli* using TOPO TA Cloning Kit (ThermoFisher). Purification of the plasmid DNA, clones for each bacterial target selection, and conventional PCR assay ensuring that the plasmid DNA contained the targeted bacterial sequence were described elsewhere (Sung et al., 2022). Finally, the abundance of each bacterial group in the 12 healthy cats was used to establish the RI using the freeware Microsoft Excel add-on Reference Value Advisor v2.1 (Geffré et al., 2011).

2.1.6. Serum parameters

Blood samples were collected from jugular veins into commercially available pro-coagulant serum-separating tubes (*Vacutainer*, Becton Dickinson, Franklin Lakes, NY, USA). After collection, the blood was allowed to clot at room temperature for 30 min. The clot was removed by centrifugation for 10 min at 2000 ×*g* using a refrigerated centrifuge. Serum samples were stored at −80 °C immediately after their preparation. Serum samples, belonging to samples taken from each cat during standard screening controls, were used to perform a complete biochemical evaluation (for the purposes of the present study, only the values of some selected serological parameters are reported). The test for determination of values of serum amyloid A (SAA), citrulline (evaluated by mass spectrometry), total protein, amylase, lipase, DGGR lipase, and cholesterol, were performed according to the accreditate laboratory's protocol (*San Marco Laboratories*, Padua, Italy). Then, serum resulting from that used for clinical purposes was used for the evaluation of bacterial LPS (ELISA Kit, MyBioSource, San Diego, CA, USA, catalog #MBS2603942), and serum zonulin (ELISA Kit (cat.nr. ab137978), Abcam). Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to measure the different parameters, according to the manufacturers' protocol. Overflow values and those under the limit of detection for every biomarker were standardized as double and half of the detection limit, respectively (Thomas, 2006). In all cases a CBC was performed from tube with K3-EDTA (ProCyte Dx, IDEXX®).

2.1.7. Histopathology, Immunohistochemistry and molecular analysis

Ten to twelve biopsy samples per cat were taken from macroscopically affected areas of the proximal portion of the small intestinal mucosa in the LG, or random in the CG, fixed in 10 % buffered formaldehyde, then embedded in a correct oriented manner in paraffin wax. The histologic examination of H&*E*-stained sections included the assessment of the histological score (Day et al., 2008), evaluated in each biopsy, and the pattern of lymphoid cells infiltration, according to a new histopathological assessment scheme for the assessment of intestinal biopsy samples from cats with chronic enteritis (Freiche et al., 2021b) and the 2016 revision of the World Health Organization (WHO) classification of lymphoid neoplasms in humans (Wolfesberger et al., 2018). According to these new guidelines, immunohistochemical expression of CD3, upregulation of STAT 5, Ki67 expression, and clonality analysis were performed, excluding from the composition of the LG group, cats that did not meet all the above criteria. For this purpose, all cases enrolled in LG group, in which H&E assessment suggested a LGITCL diagnosis, were also IHC tested by using an anti-CD3, and an anti-CD20 antibodies in attempt to identify the T-cell (1:20 dilution, F7.2.38, DakoCytomation, Santa Clara, California) or the B-cell lineage (1:400 dilution, RB-9013-P1c, Lab Vision Corporation, Fremont, California) respectively. According to Freiche et al. (2021b) proliferative index activity within the neoplastic lymphocytes population was assessed by the use of Ki-67/clone MIB-1 (1:75 dilution, MIB-1, DakoCytomation,

Santa Clara, California) as suggested, and pSTAT5 overexpression was estimate by using pSTAT5Y694/699 antibodies (Phospho-STAT5; 1:100 dilution, polyclonal antibody, Biorbyt, Cambridge, United Kingdom) on histological sections. A separate quantitative assessment of CD3 positive lymphocytes was performed in the epithelial and lamina propria compartments, in attempt to evaluate different patterns of T cells distribution in LGITCLs, ranging from massive infiltration of the lamina propria with loss of mucosal architecture, and marked epitheliotropism, characterized by lymphoid cells nests/plaques or both within the intraepithelial compartment. Finally, molecular detection of clonal B-cell receptor (BCR) and T-cell receptor (TCRγ) gene rearrangements was performed in all cases on DNA extracted from FFPE tissue slides or from frozen intestinal biopsy samples or both by multiplex PCR at the Genefast Laboratory (Forlì, Italy), as previously reported (Freiche et al., 2021b; Briscoe et al., 2011; Hammer et al., 2017). A monoclonal rearrangement is defined by the detection of a single peak and a polyclonal rearrangement is defined by the presence of >4 peaks or the absence of peaks. Oligoclonality is defined by the presence of 1 to 3 peaks on a polyclonal background.

2.2. Statistical analysis

Data were statistically analyzed using the Mann-Whitney (non-parametric test for independent samples) test to assess differences in bacterial taxa between the control group and the lymphoma group. A $p < 0.05$ was considered significant. The Mann-Whitney test was also used to evaluate the difference in serum biomarkers between the control group and the lymphoma group and a $p < 0.05$ was considered significant. Statistical analysis was performed using a software package (MedCalc® Version 22.026 © 1993–2024 Ostend, Belgium).

3. Results

Table 1 shows the changes in the main bacterial populations examined and, in particular, decrease of *Faecalibacterium* spp. and *Clostridium hiranonis* and increase *of Escherichia coli*, *Streptococcus* spp. and *Turicibacter* spp.

In Table 2 are represented serology parameters. Total proteins, amylase and cholesterol displayed a significant statistically decrease in LG respect CG and, contrary, an increased level of LPS was observed in LG group. Conversely, no statistically significant difference was observed for citrulline, serum amyloid, lipase, lipase DGGR and zonulin.

3.1. The CBC in all cases showed no significant alterations

3.1.1. Histopathology and Immunohistochemistry

In H&*E*-stained sections, all 12 cases of cats with LGITL showed a marked infiltration of both the epithelium and the lamina propria by small monomorphic lymphoid T cells. This description is different in cases of chronic lymphoplasmacytic enteropathy (LPE) where a polymorphic infiltration pattern is observed. Ten of the 12 examined LGITL cases (83.3 %), showed a concomitant intraepithelial infiltration of small lymphoid cells. Another factor that was consistently present in all examined LGTL cats (11/12 animals = 98 %) was the presence, within the crypts, of a strong lymphocytic infiltration (lymphocytic cryptitis); this aspect, also in previous research, has strongly discriminated between LGITL and LPE (Freiche et al., 2021). In 8 of 12 cats (66.6 %) in which the diagnosis of LGITL was made, pathological pictures were also observed that are typically found in chronic inflammatory disease, demonstrating that the presence of mixed pictures, LGIT and LPE is frequent and that in cats, LPE can represent a prodromal phase of LGIT. In these LGIT+LPE cases, cellular polymorphisms with mixed lymphoid and plasmacytic inflammatory infiltrates could be observed. Furthermore, in 5 of 12 cases (41.6 %), it was possible to highlight the simultaneous presence of neutrophilic cryptitis and micro-abscesses localized in some glandular lumens, with aspects of mucoviscidosis. The evaluation of the apical-basal gradient of small lymphocyte infiltration, which is characterized by a more severe apical than basal neoplastic cell infiltration at the level of the villi, was present in over 90 % (11 cats) of LGILT cats. For none of the cats of the LGILT group was it possible to observe the state of the muscularis or serous stratum, whose infiltration may represent another discriminating element with severe LPE forms. Most of the LGITL cases presented a diffuse and extensive infiltration of the lamina propria and the epithelium of the small intestine by small CD3+ T lymphocytes. In LGITL cases, the proportion of CD20+ B cells in the epithelium and lamina propria was very low. Activation of the STAT3/STAT5 pathway was assessed by phospho-STAT labeling. Phospho-STAT3 (pSTAT3) was not expressed, whereas phospho-STAT5 (pSTAT5) was positive in 100 % of LGITL tumor cells.

Table 1
Changes in the main intestinal bacterial populations in cases and controls.

	Median CG	Median LG	P
Escherichia coli	34,655	67,586	0,0045*
Faecalibacterium spp	67,903	59,708	0,0153*
Clostridium Hiranonis	64,584	0,8647	0,0433*
Streptococcus spp	30,645	46,689	0,0003*
Turicibacter spp	46,544	55,258	0,0056*

Statistical significance: *P < 0,05.
CG: control group; LG: lymphoma group.

Table 2
Modifications in biochemical parameters.

	Median LG	Median CG	P
SAA	33,50	32,00	0,3404
Citrulline	52,00	54,00	0,7223
Total Protein	61,00	74,00	**0,0092***
Amylase	1017,00	1605,00	**0,0092***
Lipase	29,00	68,00	0,2100
Lipase DGGR	72,00	70,00	0,9697
Cholesterol	112,00	150,00	**0,0112***
LPS	0,585	0,295	**0,0024***
Zonulin	7,03	2,75	0,1011

Statistical significance *P < 0,05.
LG: lymphoma group CG: control group.
SAA: Serum Amyloid A; LPS: Lipopolysaccharide.

4. Discussion

The aim of the present study was to investigate possible diagnostic and prognostic parameters in cats with low-grade intestinal t-cell lymphoma. Selected fecal bacteria and the above-mentioned serum markers were investigated in cats presenting LGITCL, and compared with healthy controls.

Studies showed that there are no significant differences between the diagnosis made on well-performed endoscopic biopsies and full-thickness laparoscopic biopsies for LGITCL(Marsilio et al., 2023). However, research in this area is aimed at finding new serological and fecal markers that may be indicative of the diagnosis of LGITCL with respect to chronic inflammatory forms, such as lymphoplasmacytic enteritis (LPE); the evaluation of some parameters as possible candidates for a non-invasive diagnosis of LGITCL would be desirable for prognostic purposes but above all to follow cats affected by this frequent intestinal neoplasia, in the *follow-up* after diagnosis, and to test their response to chemotherapy protocols. In our study group, as expected, the condition of LGITCL was accompanied by a significant variation in serum levels of total protein, cholesterol, amylase and LPS; more precisely, total protein, cholesterol and amylase were reduced in LG, while LPS levels were increased compared to CG. These parameters, typically indicative of a "*leaky gut*" condition, highlight how the clinical symptomatology of LGITCL are due by serious damage to the intestinal barrier. There is also

loss of functionality of the mature enterocytes, which are located at the apical level of the villi, with a strong reduction of their enzymatic activities. The altered permeability of the intestinal barrier is linked both to the infiltration of lymphoid cells through the epithelium, with a consequent rupture of the intercellular junctions, and to a decrease in functional junctions, linked to the mitochondrial damage of the enterocytes caused by intestinal dysbiosis (Santinelli et al., 2023). This condition can cause an increase in bacterial translocation through the intestinal epithelium, with an increase in serum LPS (Rossi, 2022). Bacterial LPS can be considered an indirect indicator of bacterial translocation and has some pro-inflammatory activity. Indeed, when LPS is recognized by the Toll-like receptor 4, it is activated, leading to the synthesis of proinflammatory mediators (cytokines and chemokines) (Vaure and Liu, 2014). Interestingly, LPS increase in LG cats confirms the expected loss of intestinal mucosal barrier integrity, as also demonstrated by histopathology. Pathologists described different patterns of cell distribution in LGITCL (i.e., massive infiltration and deletion of the lamina propria morphology; marked epitheliotropism; "nests"/ "plaques" of lymphoid infiltration in the intraepithelial compartment) (Marsilio et al., 2023), in all cases the lesions seem to originate in the apical part of the villi and then expand through the lamina propria with a transmural appearance. This new view of LGITCL as an "*up-bottom*" lesion compared to the previous idea that LGITCL was a "*bottom-up*" lesion originating from a chronic expansion of the GALT, appears very interesting and highlights even more the central role of the damage of the epithelial barrier, in the progression/prognosis of the disease (Freiche et al., 2021a; Marsilio et al., 2023). SAA is one of the major acute phase proteins whose concentration may increase up to 1000-fold in inflammatory status in cats (Tamamoto et al., 2008). In cats, SAA concentration increased not only in inflammatory diseases but also in neoplastic and non-inflammatory diseases, such as diabetes mellitus and hyperthyroidism (Tamamoto et al., 2008). In cats as in humans, SAA concentration is reported not only as a marker for the presence of inflammation, but also as a prognostic indicator. Recent study (Tamamoto et al., 2013) showed that higher serum levels of SAA in cats are inversely correlated with median survival time, and could represent a prognostic marker in cats with various diseases regardless of diagnosis. Previous studies showed a positive correlation between SAA levels in cats with CE, suggested a significant difference in SAA concentration in LG cats compared to control cats. During chronic pathologies as LGITCL there are several reasons why SAA concentration could function as a prognostic marker, suggesting a poorly controlling of disease (den Hartigh et al., 2023). Chronic intestinal inflammation has the potential to increase the risk of developing LGITCL, and lymphoma-associated lymphoplasmacytic enteritis (LPE) has been described in over 60 % of LGITCL (Moore et al., 2005; Moore et al., 2012; Kiupel et al., 2011; Briscoe et al., 2011; Lingard et al., 2009; Carreras et al., 2003). Different studies hypothesized that LPE may precede or promote gastrointestinal neoplasia (Lingard et al., 2009; Mahony et al., 1995; Fondacaro et al., 1999; Waly et al., 2005; Castro-López et al., 2018; Hart et al., 1994). Recently, it has been observed that some cats diagnosed with LGITCL may develop large cell lymphoma over time (Wright et al., 2019); since this type of lymphoma is independent of chronic inflammation, it is hypothesized that in many cases the onset and development of the neoplasm may derive from distinct clones of cells independent of inflammation (Moore et al., 2012). Citrulline is considered an indicator of enterocyte mass and intestinal absorption in human medicine; despite the absence of statistical significance, our results showed that the value of citrulline in LG resulted decreased compared to the controls, indicating a possible reduction of functioning enterocytes, in agreement with the histological analysis of a strong epithelial infiltration and the serological increase of LPS. Amylase's value was statistically decreased in LG respect CG because the small intestine is a source of serum amylase activity. Lipase decreased, but non statistically significative and within the reference values, in cats with lymphoma compared to control cats whereas the specific pancreatic Lipase DGGR remains unchanged. Finally, zonulin is a tight junction protein linked to the integrity of the intestinal barrier, appears increased in the course of LGITCL, compared to controls even if in this case the increase was not statistically significant. Previously we had observed a similar behavior also in dogs with intestinal lymphangiectasia (IL) (Rossi et al., 2021) highlighting how this protein can be a good marker following damage to the mucosa. Tight junctions (TJs) surround the apical portion of enterocytes and regulate the paracellular permeability of solutes. TJs are multiprotein complexes, and the intracellular domain of these proteins interacts with cytosolic proteins, the zonula occludens (ZO) proteins, which anchor the transmembrane proteins to the actin cytoskeleton. The interaction between the tight junctions and the actin cytoskeleton is critical for maintaining the tight junction structure, enabling regulation of the paracellular pathway (Kucharzik et al., 2001). One of the modulators of these TJs, is the toxin zonula occludens (Zot) (Di Pierro et al., 2001; Fasano, 2008). Serum zonulin is overexpressed in pathological conditions that alter intestinal permeability (Ramezani Ahmadi et al., 2020). In humans, zonulin expression has been correlated with several pathologies and linked as a biomarker of intestinal permeability (Fasano, 2020). In all intestinal and metabolic pathologies in which an increase in the permeability of the intestinal barrier is observed, due to an early disassembly of the enterocytes, a release of zonulin is observed which passes freely into the serum and directly into the intestinal lumen (Sturgeon and Fasano, 2016). One of the factors related to the disassembly of the *Zonula occludens* protein complex is linked to the reduction of intracellular ATP and mitochondrial damage (Rossi, 2022; Santinelli et al., 2023). An altered pattern of gut microbial flora composition, directly activate the apoptosis machinery of enterocytes, via mitochondria alteration (increasing intrinsic and extrinsic apoptosis pathways), and apical junctional complex (AJC) morphology modification (Santinelli et al., 2023). Bacterial mucosal colonization has been investigated as a driver of neoplastic transformation in humans, dogs, and cats (Marsilio et al., 2023). Although a statistically significant association of mucosa-invading and intravascular bacteria has been found in intestinal large cell lymphomas in cats, no association between LGITL and bacterial invasion has been reported (Hoehne et al., 2017). Dysbiosis in humans and animal models of LPE has been found to promote inflammation and malignant transformation, especially the development of colorectal cancer (Zou et al., 2018). The role of dysbiosis in CE of cats is poorly understood. Actually, the DI is validated also for cat (Sung et al., 2022; Giordano et al., 2024; Benvenuti et al., 2024). The dysbiosis index (DI) is a quantitative PCR-based assay that can be used to assess the feline (Sung et al., JFMS 2022) or canine (AlShawaqfeh M et al., FEMS 2017) fecal microbiome in individual patients. It is currently the only analytically validated assay to assess the fecal microbiome and has been used in various published clinical studies. The DI quantifies the fecal abundance of seven bacterial taxa as well as the total bacterial abundance. These bacterial taxa are commonly altered in chronic enteropathies (CE) and after broad-spectrum antibiotic use. The DI provides reference intervals for these bacterial groups and additionally calculates a single number that expresses the extent of intestinal dysbiosis. The DI correlates negatively with species richness, i.e., a higher DI indicates lower microbial diversity.

Although the DI was found to be discriminatory in identifying cats with enteropathy related to CE/IBD and LGILT, compared to healthy cats (in fact cats with CE/ LGILT had a significantly higher DI than healthy cats); the DI did not differ between cats with IBD and cats with LGILT. For this reason, in our work, we preferred to focus our evaluation on the trend of some bacterial species that are normally more altered in cats with CE/LGILT compared to healthy cats, to analyze their trend in the cases of LGILT that we enrolled, rather than simply calculating the DI.

Although some Authors indicate that there are no substantial differences between the dysbiotic patterns of cats with LGITCL and LPE (Marsilio et al., 2019), it is undeniable that both in inflammatory and neoplastic situations, an alteration of the normal microbiota observed in

healthy cats is produced. In our study, LG showed changes in the main bacterial populations examined and, in particular, a decrease of *Faecalibacterium* spp. and *Clostridium hiranonis* and an increase *of Escherichia coli*, *Streptococcus* spp. and *Turicibacter* spp. were documented. Regarding the differences observed in fecal bacteria, is particularly interesting a reduction of *C. hiranonis* which, by modifying the concentration of secondary bile acids, but also the SCFAs production, can, together with the previously discussed reduction of lipase and amylase determine a hypo- absorption worsening the clinical profile of cats with LGITCL. Whether cats with a low abundance of *C. hiranonis* might have abnormal bile acid metabolism and a higher risk of harboring more pathogenic bacterial groups needs to be evaluated in further studies (Sung et al., 2022). In addition, dysbiosis and perturbations of fecal metabolic profiles have been reported in dogs with inflammatory bowel disease (Galler et al., 2022; Minamoto et al., 2019) as well as in other species with intestinal neoplastic conditions (Quaglio et al., 2022).

One limits of the research is that it was not possible to determine folate and cobalamin levels. Cobalamin decreases in feline patients with lymphoma and the evaluation may be useful for a possible therapeutic implementation in deficient subjects and less for diagnostic purposes (Jugan and August, 2017).

5. Conclusions

To date, there is not a single diagnostic test which is non-invasive or based on histopathological and immunohistochemical evaluation of intestinal biopsy, to reliably differentiate LPE from LGITCL. The combination of clinical data (e.g., age, duration of clinical signs), imaging, laboratory data, histopathology, immunohistochemistry, and clonality testing appear to be the best approach to reach a final diagnosis of feline LGITCL. However, classification schemes and diagnostic tests are expected to evolve over time and ultimately improve the accuracy of diagnostic tests and more importantly, treatment options for affected cats. Other biomarkers are also being developed and tested for sensitivity and specificity. In the present study, total proteins, cholesterol, amylase, and lipase levels resulted decreased in cats suffering from LGITCL when compared to healthy cats. Conversely, serum concentrations of bacterial LPS, but also zonulin and SAA (even if the last two not significantly) were higher in LG cats, necessitating a further investigation of their possible application as a prognostic marker in cats with LGITCL. Significant differences were also found in the selected bacteria investigated. The present data need to be confirmed and enriched with future findings for a better understanding; however, these results represent important steps toward the possible identification of new markers of greater intestinal barrier damage and worse patient/recovery prognosis.

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CRediT authorship contribution statement

Alessandra Gavazza: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sara Mangiaterra:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Investigation, Formal analysis, Conceptualization. **Livio Galosi:** Writing – review & editing, Writing – original draft, Visualization, Data curation, Conceptualization. **Alessia Dottori:** Writing – review & editing, Writing – original draft, Visualization, Validation, Data curation, Conceptualization. **Lucia Biagini:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Data curation, Conceptualization. **Graziano Pengo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Data curation, Conceptualization. **Jan Suchodolski:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Data curation, Conceptualization. **Matteo Cerquetella:** Writing – review & editing, Writing – original draft, Visualization, Validation, Data curation, Conceptualization. **Giacomo Rossi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

None.

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