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Abstract

Among the intestinal tumours of hematopoietic origin, lymphoma is the most common in the dog. The multicentric lymphoma is more common than the intestinal form, while the alimentary lymphoma usually accounts for 7% of all canine lymphomas and 5-7% of all gastrointestinal neoplasms. Recent studies have shown that canine intestinal lymphomas are to great extent of T-cell origin. Affected dogs most often present with a recent history of non-specific signs, like vomiting, diarrhea, anorexia and lethargy. The dogs involved are most commonly adult or old subjects, with an average age of 6,7 years. The most commonly involved breeds are Boxer, Shar-Pei and Golden Retriever. In light of the clinical and symptomatic overlap between IBD and enteropathy-type lymphoma, patients are often submitted to endoscopic examination. The endoscopic examination of the small bowel in the lymphoma group showed an aspect referred by gastroenterologists as "clobberstone appearance" of the intestinal mucosa: the submucosal edema and the ulcerative disease with crisscrossing of the ulcers creates a pattern characterised by multiple, similarly-sized rounded densities that rise above a flattened plane, which has been likened to roads paved by multiple similarly-sized 'cobble' stones. In dogs with this endoscopic aspect, the cytological examination alone has shown in our study a good diagnostic accuracy in the differential diagnosis between lymphoma and IBD. In fact, we argue that a cytological examination of squash-prep samples is a valuable method for the detection of a infiltrating enteropathy. In our study, we evaluated the samples using an interpretative algorithm based on a reduced number of some selected cytomorphological clues. These criteria were: 1) presence of lymphoid blast cells, 2) fine

structural change of the lymphoid blasts's membrane, 3) presence of lympho-glandular bodies.

The most outstanding result, was the higher diagnostic accuracy of squash-prep cytology compared with endoscopic biopsy histology. Histopathology and immunohistochemistry are generally considered useful in the differential diagnosis between IBD and lymphoma. In our study however, although the evaluation of histopathologic results is not within the aims of the present work, the diagnostic performances of histopathology on endoscopic biopsies didn't show a better accuracy better than those of cytology alone.

Introduction

The small intestine (SI) works as an interface between the external environment and the body, and is both an absorptive surface and a barrier; it must digest and absorb nutrients while excluding antigens and microbes and eliminating fecal waste.

The alimentary system varies in its architecture and function among animal species. Pet carnivores tend to develop alimentary neoplasia far more often than herbivores. This can be partially explained by their long life span and an exposure to environmental, cancerogenic factors similar to those of humans beings. Various types of neoplasms occur in the gastrointestinal system of domestic animals. Intestinal neoplasms are diagnosed most frequently in dogs and cats.

1. ANATOMY AND PHYSIOLOGY

Anatomical regions

The SI can be likened to a convoluted tube, extending from the pylorus of the stomach to the ileo-colic valve. It exists a continuity with the external environment, proximally from the oral cavity via the esophagus and stomach, and distally to the anus via the large intestine (Buddington RK, 1996; Kararli TT, 1995; Snipes RL and Snipes H, 1997).

1.1 Duodenum

The first part of the SI, the duodenum, comprises about 10% of its total length. It extends dorsally from the pylorus and to the right, at the level of the 9th intercostal space, where is maintained in its position by the hepatoduodenal ligament. It turns then caudally into the descending duodenum and again at the caudal flexure near the pelvic brim. It is in close association with the common bile duct and the head and right limb of the pancreas, which lie in its mesentery.

The common bile duct and one pancreatic duct enter the duodenum via the major papilla. In dogs, an accessory pancreatic duct often enters at a minor papilla more distally and slightly more ventrally, but there is a range of variations in the actual number of ducts and their drainage pattern from the pancreas. The papillae are notable endoscopic landmarks in dogs.

The distal duodenal flexure, where the duodenum courses to the left side of abdomen is often at the limit of reach of a standard 1-meter gastroscope (except in small dogs). In dogs the anti-mesenteric side of the duodenum is marked by a line of whitish, mucosal depressions signifying the presence of specialized lymphoid areas, the Peyer patches. Secretory Brunner glands and annular mucosal folds are features of the human proximal duodenum, but are not present in dogs. After the distal duodenum flexure, the ascending limb of the duodenum crosses the midline and ends at the level of L6 close to the root of the mesentery near the left kidney, with a mesenteric attachment to the colon, the duodeno-colic ligament.

1.2 Jejunum

The middle part of the SI, the jejunum, arises as an indistinct structural and functional transition from the duodenum and forms the majority of the SI. The jejunum is loosely suspended in the middle of the peritoneal cavity in a dorsal mesentery, forming mobile loops, and is potentially palpable throughout its length in cooperative and non-obese patients. The mesentery is normally a continuous sheet that is folded to allow the SI to loop within the peritoneal cavity, unlike in humans where segments of the duodenum (and colon) are retroperitoneal. The mesentery carries the vascular, lymphatic, and nervous connection between the SI and the rest of the body.

Defects in the mesentery, most often traumatic in origin, can allow internal hernia formation and small intestinal incarceration. An outpouching of the dorsal mesentery of the stomach forms the greater omentum. This structure functions as a protective, immuno-

logic organ, having the ability to migrate to sites of intraperitoneal inflammation and potentially prevents leakage from an intestinal perforation and seal off pockets of infection.

1.3 Ileum

The ileum constitutes approximately the final 30 cm of the SI. The transition from jejunum to ileum in humans is based on changes in diameter, color, and the presence of Peyer patches; in dogs the distinction has been arbitrarily based on the extent of attachment of the ileocolic ligament. In fact, the basic structure of the ileum is not different from the rest of the SI and there's no clear microscopical demarcation from the jejunum. However, the ileum does have in dogs some unique functional characteristics, such as the absorption of bile salts and cobalamin. It is also a site of dense lymphoid follicle expression. Merkel diverticulum, a remnant of the embryonic omphalo-mesenteric duct, found in the ileum of approximately 2% of people and a potential source of bleeding, obstruction, intussusception, and volvulus, is not reported in dogs. The ileum ends at the ileocolic valve in close association with the cecocolic junction.

2. Blood Supply, Lymphatic Drainage, innervation

Blood supply to the proximal duodenum is provided by the celiac artery, whereas its anastomoses between its cranio-pancreatico-duodenal branch and caudo-pancreatico-duodenal branch represent the major blood supply to the remainder of the SI and proximal colon, anastomosing distally with the caudal mesenteric artery. The celiac artery forms an arcade along the mesenteric border of the jejunum and ileum, with a short anti-mesenteric ileal branch. Its branching nature is an important consideration when assessing the viability of lengths of SI during surgical resection and end-to-end anastomo-

sis. The venous drainage of the whole SI is ultimately to the liver via the hepatic portal vein. There exist multiple embryonic vessels linking portal venous drainage and the systemic venous system (i.e., via ovarian veins, caudal vena cava, and esophageal veins) but they only become a functional shunt as a consequence of chronic portal hypertension. Lacteals in the villi drain via intestinal lymphatics in the mesenteric lymph nodes and then in the cisterna chyli and the thoracic duct. Vagal and sympathetic innervation coordinate with the intrinsic enteric nervous system and enteric hormones to regulate SI motility and function.

3. Gut-associated Lymphoid Tissue

The GI tract is the largest immunologic organ in the body, and the SI comprises a large component of the mucosal immune system. Within the SI, the Peyer's patches act as inductive sites and are covered with a specialized epithelium containing microfold (M) cells, which sample luminal antigens. Activated lymphocytes migrate via mesenteric lymph nodes to the circulation, from where they home to their effector sites, the *lamina propria* and epithelium.

The GI immune system is compartmentalized into:

- a) afferent or inductive sites, where antigen-presenting cells (APCs) prime naïve T and B cells to initiate the immune response, either by processing and presenting local antigens or by migration from the *lamina propria* (LP), an important site of antigen sampling.

b) efferent or effector sites, where the antibody and T-cell-mediated response is mounted after extravasation, retention, and further differentiation of the lymphocytes.

The afferent arm of the GI immune system comprises Peyer patches (PPs), isolated lymphoid follicles (ILFs), and mesenteric lymph nodes (MLNs), while the efferent arm comprises lymphocytes located in the *lamina propria* and epithelium; cryptopatches, loosely organized clusters of approximately 1000 cells located at the base of the intestinal crypts.

The intestinal *lamina propria* contains plasma cells, T cells and putative DCs (Elwood CM et al, 2006; German AJ et al, 1999). Plasma cells are predominantly IgA⁺ and are concentrated within the crypt regions in the small intestine and deep lamina propria in the colon; fewer of them are found within the villi and superficial lamina propria of the colon and there is a progressive decline in the density of IgA⁺ plasma cells from the duodenum to the ileum (German AJ et al, 1999; Vaerman JP and Heremans JF, 1969; ; Willard MD et al, 1981). By contrast, CD4⁺ and CD8⁺ T cells within the canine *lamina propria* are concentrated toward the tips of the villi, with no apparent differences in proximal to distal small intestinal distribution (German AJ et al, 1999; Elwood CM and Garden OA, 1999). The ratio of CD4⁺/ CD8⁺ T cells is approximately 60:40 in the *lamina propria* and 15:85 in the epithelium (Elwood CM and Garden OA, 1999). Subtractive analysis suggests that a population of CD3⁺CD4⁻CD8⁻ cells exist in the canine villus epithelium, thought to represent $\chi\delta$ T cells. Immunohistochemical studies have suggested that $\alpha\beta$ and $\chi\delta$ IELs are present in approximately equal numbers in the canine duodenum, jejunum, and ileum (Ger-

man AJ, et al, 1999). A significant number of lymphoid cells within the canine *lamina propria* are CD45R, raising speculation that a proportion of the T cells found within this compartment are naïve, in contrast with the predominantly effector/memory T-cell phenotype observed in the human *lamina propria* (German AJ et al, 1999).s in the villus LP (Elwood CM and Garden OA, 1999; German AJ et al, 1998). However, the strongest MHC class II expression occur within the Peyer patches and some expression of this antigen is also apparent within the epithelium, particularly within the ileum (Elwood CM and Garden OA, 1999; German AJ et al, 1998).

4. NON- NEOPLASTIC DISEASES OF GI TRACT: IBD

Canine inflammatory bowel disease (IBD) is characterized by persistent gastrointestinal signs, histological evidence of mucosal inflammation, and general responsiveness to immunotherapeutic intervention (Strombeck DR, 1979). Clinical signs are highly variable, with disease severity resulting from numerous factors, including the presence or absence of active mucosal inflammation, organ(s) of involvement, physiological (e.g., anemia or vitamin deficiencies) consequences, and empirical therapies (Jergens AE *et al* 1992; Guilford WG 1996; Jacobs G *et al* 1990; Jergens ,1999).

Disease activity caused by IBD denotes the frequency and severity of clinical signs, such as vomiting and diarrhea, which occur as a consequence of diffuse mucosal inflammation.

Assessment of IBD activity depends at present on a compendium of clinical, radiographic, endoscopic, and histological criteria that vary widely from one clinician to the other

(Jergens AE *et al* 1992; Guilford WG, 1996; Jacobs G *et al* 1990; Jergens,1999; Strombeck DR, 1979). The development of a standardized scoring index, such as those used in human IBD, would be useful in the management of affected animals, both as a measure of initial disease severity and to assess treatment responses (Jergens AE.,1999).

4.1 Immunological and Inflammatory Mediators

Recent advances in immunohistochemical techniques have identified imbalances in mucosal immune cells in canine IBD (German AJ, 2001; Jergens AE *et al*, 1999; Jergens AE *et al*, 1996; Stonehewer J *et al*, 1998; Elwood CM and Garden OA. 1999; Locher C *et al*, 2001; Jergens AE *et a*, 2001). Immune-cell subset alterations involve both B and T lymphocyte populations and are broadly suggestive of pro-inflammatory changes within the mucosa, as compared to healthy dogs and dogs diagnosed with other causes of gastroenteritis.

Some methods for evaluating canine IBD activity on the basis of nitric oxide production and altered mucosal cytokine messenger ribonucleic acid (RNA) expression have been described (Gunawardana SC *et al*, 1997; Jergens AE *et al*, 1998; German AJ *et al*, 2000; Ridyard AE *et al*, 2002; Fugiwara S *et al*, 2002; Jergens AE *et al*, 2003).

Furthermore, a strong association between serum CRP concentrations and a numerical canine IBD activity index (CIBDAI) has been noted (Jergens AE *et al*, 2003). Preliminary data also suggest that perinuclear anti-neutrophil cytoplasmic antibodies might be useful as a serological marker of canine IBD (Allenspach K *et al*, 2003). To date, clinical research investigations (predominantly performed in the dog) validate disturbances in

mucosal immunity in dogs with IBD. Unfortunately, only a few of these immunological parameters have been correlated to the severity of clinical disease activity. Moreover, the lack of consistent endoscopic abnormalities and the absence of standardized histological criteria for diagnosis of IBD hinder use of these indices as reliable markers of intestinal inflammation. Recently, sonographic findings including focal bowel wall thickening, loss of an organized definition to the architecture of the intestinal wall, and mesenteric lymphadenopathy have been shown to be relevant in staging feline IBD (Baez JL *et al* 1999). However, similar radiographic studies have not been performed in dogs with IBD. Given these limitations and previous experiences with human IBD indices, the use of gastrointestinal signs and simple parameters of inflammation (such as the acute-phase response) appear to be the best method of clinical assessment of canine IBD. Methods for evaluating canine IBD activity on the basis of nitric oxide production and altered mucosal cytokine messenger ribonucleic acid (RNA) expression have been described [see Figure 1] (Gunawardana SC *et al*, 1997; Jergens AE *et al*, 1998; German AJ *et al*, 2000; Ridyard AE *et al*, 2002; Fugiwara S *et al*, 2002; Jergens AE *et al*, 2003).

Table
Canine Inflammatory Bowel Disease Immunopathological Summary

Parameter [*]	Immunological Abnormality [†]	References
Mucosal B lymphocytes	↓ or ↑ IgG cells in small intestine ↑ IgA, IgG cells in colon ↑ plasma cells in colon	54, 56 55 57
Mucosal T lymphocytes	↓ CD3 ⁺ cells in small intestine ↑ CD3 ⁺ , CD4 ⁺ , TCRαβ ⁺ cells in small intestine ↑ CD3 ⁺ , CD8 ⁺ cells in colon ↓ TCRγδ ⁺ IEL	56 54 55 60
Mucosal mast cells	↓ or ↑ mast cells in small intestine	54, 59
Nitrate in lavage	↑ colonic luminal nitrite and IgG	61
Mucosal iNOS	↑ iNOS in small intestine and colon	62
Mucosal cytokines	Balanced Th1:Th2 mRNA expression in small intestine or colon	63, 65, 66
Acute-phase proteins	Predominant Th1 mRNA expression in colon ↑ serum concentration of CRP	64 67, 68
Antineutrophil antibody	↑ serum pANCA concentration	68

^{*} iNOS=inducible nitric oxide synthase
[†] ↓=decreased; ↑=increased; IgG=immunoglobulin G; IgA=immunoglobulin A; CD=cluster of differentiation; TCR=T cell receptor; IEL=intraepithelial lymphocyte; Th=T helper; mRNA=messenger RNA; CRP=C-reactive protein; pANCA=perinuclear antineutrophil cytoplasmic antibody

Figure 1 JOURNAL of the American Animal Hospital Association. November/December 2004, Vol.40

4.2 Simple Canine IBD Activity Index

Recently, a prospective clinical study evaluating the use of a simple scoring index (CIBDAI) for assessment of canine IBD activity at diagnosis and following medical therapy has been published. Using this system, six prominent gastrointestinal signs were scored 0 to 3 based upon the magnitude of their alterations from normal in a given IBD case. These scores were then summed, yielding a total cumulative CIBDAI score, which classified the disease as clinically insignificant, mild, moderate, or severe. The CIBDAI score was also correlated to the severity of histological lesions and the serum concentration of CRP, an acute-phase reactant; these data showed that CRP levels were dramatically elevated in 58 IBD dogs at the time of diagnosis, particularly in those dogs (n=28) having CIBDAI scores reflective of moderate to severe IBD (Jergens AE *et al*,

2003). Additionally, both the CIBDAI score and the CRP concentration decreased in dogs following successful medical (e.g., immunosuppressive drug therapy and dietary management) therapy for their disease .

It is noteworthy that dogs having diverse, non-IBD, enteric (e.g., alimentary lymphoma, acute pancreatitis, mucosal histoplasmosis) and extra-alimentary tract disorders (e.g., multicentric lymphoma, inflammatory hepatic disease, severe cutaneous parasitism) also had elevations of serum CRP. However, these elevations were significantly higher than the CRP concentrations found in IBD dogs, suggesting the presence of different acute-phase responses to severe localized inflammation in a variety of tissues.

The accumulated observations derived from this investigation support the CIBDAI as a reliable measure of clinical signs of inflammation in dogs with IBD.

It was also observed that both CIBDAI and CRP decreased in successfully treated dogs, making a measure of CRP level suitable for laboratory evaluation of the effect of therapy in these patients.

5. NEOPLASTIC DISEASES OF GI TRACT

5.1 Incidence and risk factors

Reports vary, but overall intestinal tumors are rare in dogs and cats (Patnaik AK et al, 1976). In a survey of insured dogs in the United Kingdom, a standardized incidence rate of 210/100,000 dogs was reported for alimentary tumors. This accounted for 8% of all tumor submissions (Dobson JM et al, 2002).

Regarding specific tumor types, lymphoma represents 6% of all canine tumors and is the most common intestinal tumor in most reports (Dorn CR et al, 1968; Crawshaw J, et al.,1998). Each tumor will be described in detail in the next chapter. Adenocarcinoma is the second most frequent tumor in both species, followed by mast cell tumors in cats and leiomyosarcomas or GISTs in dogs.

As with many cancers, incidence of intestinal neoplasia increases in older dogs.

Dogs are also usually middle aged or older, with mean ages most often between 6 and 9 years, possibly older (12 years) for dogs with leiomyosarcoma (Patnaik AK et al, 1977; Couto CG et al, 1989; Kapatkin AS et al, 1992). There is a slight sex predilection for male dogs to develop intestinal tumors in some studies. Some studies report a near equal incidence among male and female dogs (Miura et al, 2004; Myers NC and Peninck DG, 1994; Valerius et al, 1997), although in one study 76% of dogs with intestinal adenocarcinoma were male (Paoloni et al, 2002). Males also appear overrepresented in smooth muscle tumors, comprising 82% of dogs with GI leiomyomas (Frost D et al, 2003) and 76% of dogs with leiomyosarcoma. Additionally, 90% of dogs with GI lymphoma were male (Couto CG. et al, 1989).

In dogs, few studies of intestinal neoplasia report an over-representation of specific breeds. Large breed dogs in general constituted most cases in a series of smooth muscle tumors. Collies and German Shepherds are over-represented in some reports for intestinal tumors, especially adenocarcinoma and rectal carcinoma and polyps (Seiler RJ, 1979). It is interesting to note, however, that in 104 benign and malignant tumors diagnosed in a cohort of military working dogs (German Shepherd dogs and Belgian Malinois), only one (a leiomyosarcoma) was intestinal (Peterson et al, 2000). Mast cell tumors have been reported primarily in Maltese, among other miniature breeds. Although these reports came from Japan where small breeds are popular, over 50% of reported cases in two series were in Maltese dogs, with a male predominance (Ozaki et al, 2002; Takahashi et al, 2000).

Helicobacter pylori infection is associated with increased risk of gastric cancer in humans, although such association has not been confirmed in domestic animals. Multiple gastroduodenal adenocarcinomas and a rectal adenoma were found in a cougar with concurrent *Helicobacter*-like organisms and spirochetes.

Finally, lymphoma (although not specifically intestinal) has been reported in a dog 4 weeks after initiation of cyclosporine and ketoconazole therapy for anal furunculosis (Blackwood et al, 2004). An association between cyclosporine use in human transplant patients and the development of lymphoma is known.

5.2 Pathology and Natural Behavior

Epithelial, mesenchymal, neuroendocrine, and discrete/round cell neoplasia can all be found in the intestinal tract. Most small intestinal neoplasia in dogs are malignant, whereas more distal areas of the GI tract tend to develop a more benign disease.

Tumors include lymphoma, leiomyosarcoma and carcinoids. There are scattered case reports of uncommon tumors, such as extramedullary plasmacytoma, extraskeletal osteosarcoma, and hemangiosarcoma.

5.2.1 Lymphoma

Lymphoma is a common neoplasm in dogs and is anatomically classified into multicentric, mediastinal, alimentary and extranodal form. The alimentary form is uncommon in dogs accounting for only 5% to 7% of all lymphomas (Couto et al, 1989; Theilen et al, 1987). The gastrointestinal form of lymphoma is second in frequency only to the multicentric form (Theilen GH et al,1987). Despite gastrointestinal lymphoma being the most common extranodal form of lymphoma, the veterinary literature regarding the clinical outcomes of dogs afflicted with this disease is sparse. Much of the literature currently available on lymphoma in dogs is either centered on the multicentric form or does not distinguish between different anatomical forms (Kellen ET et al, 1993; Baskin CR et al, 2009). As a consequence, direct interpretation of the information regarding the gastrointestinal form of lymphoma is exceedingly difficult. In fact, only one published case series directly examining clinical cases of canine gastrointestinal lymphoma has been pub-

lished, and results of chemotherapy were discouraging, with most dogs succumbing to the disease or being euthanized 3 to 14 weeks after diagnosis (Couto et al, 1989).

Currently, no clear consensus exists regarding sex predilection for lymphoma. In some studies, the percentage of males affected with gastrointestinal lymphoma was higher than that of females, while no difference found in other studies (Keller ET et al, 1993; Price GS et al, 1991). Arguably, the low case number of these studies confere them a weak statistical power.

The clinical presentation of these dogs may range from nonspecific signs such as anorexia and lethargy to severe vomiting and hemorrhagic diarrhea (Couto CG et al, 1989; Ozaki K et al, 2006; Miura T et al, 2004). It is generally believed that full thickness biopsy of the intestine is necessary to diagnose alimentary lymphoma as the lesion are usually deep-seated and invade the serosa. A number of protocols is relatively successfully used at the moment in the treatment of multicentric lymphoma (Keller ET et al, 1993; Price GS et al, 1991; Halliwell B and Gutteridge JM. 1990).

Reported median survival times range from 6 to 12 months, with complete remission achieved in 60% to 90% of cases (Vail DM et al, 1991). Canine gastrointestinal lymphoma has been treated empirically with many of these same protocols (Ozaki K et al, 2006; Miura T et al, 2004). Couto and colleagues documented a poor clinical response to chemotherapy in dogs with gastrointestinal lymphoma, reporting that all but one dog died or was euthanized within 14 weeks after diagnosis (Couto CG. Et al, 1989). This poor outcome has been attributed to the use of single-agent therapy and the lack of good multi-agent protocols at the time of the study of Frank et al. (2007), hazard analysis showed that dogs treated with multi-agent therapy had a death rate of 0.40. This

rate was not significantly different from that of dogs treated with prednisone alone ($P=0.074$). Classification and diagnosis of canine lymphoma with regard mainly to GI lymphoma will be addressed in a separate chapter below.

5.2.2 Epithelial Tumors

The second most common intestinal tumors are those of epithelial origin. Colon and rectum are the most common sites in dogs, with rectum adenocarcinoma being more common than colon adenocarcinoma (Church et al, 1987).

Histologic descriptors for carcinoma of the intestine include adeno- (forming glands), mucinous (>50% mucin), signet ring (>50% of cells have intracellular mucin), and undifferentiated or solid (no evidence of gland formation). Grossly, colorectal adenocarcinomas may demonstrate a pedunculated (especially in the distal rectum), cobblestone (middle rectum), or annular (middle rectum) appearance, which may relate to behaviour and prognosis (Prater et al,2000).

Carcinoids are rare tumors of neuroendocrine origin that have been infrequently reported in dogs (Giles R. et al, 1974; Patnaik AK et al, 1980; Albers TM et al, 1998; Sako T et al, 2003; Morrell CN et al, 2002) and tend to metastasize. In particular, in one study of canine intestinal neoplasms, it was shown that half of the metastases in the liver were from carcinoids, despite these tumors numbering only 4 out of a total of 35 (Patnaik AK and Hurvitz AI, 1980.). A similar aggressive pattern has been described in humans (Oberberg K, 2001). Carcinoid cells arise from enterochromaffin cells of the intestinal mucosa and contain secretory granules that may contain substances such as 5-hydroxytryptamine (serotonin), secretin, somatostatin, and gastrin, among others (Head

KW et al, 2002) . IHC for cytokeratin and for secretory substances such as serotonin may be positive, and serum concentration of serotonin has been documented at 10-fold the normal range in one dog with a carcinoid (Sako T et al, 2003).

The prognosis for dogs with intestinal carcinoma or adenocarcinoma is considered poor, with survival ranging from 4 to 10 months (40% 1-year survival) when surgically treated (Crawshaw J et al, 1998; Paoloni MC et al, 2002; Birchard SJ et al, 1986). This poor survival rates are likely a result of a high metastatic rate. 40 to 50 percent of intestinal epithelial malignancies metastasize to local lymph nodes, while 30 to 40 percent metastasize to distant sites (Patnaik AK et al, 1980; Crawshaw J et al, 1998; Kapatkin AS et al, 1992). The clinical importance of lymph nodal metastatic disease is further highlighted by a report documenting a median survival time of dogs treated with surgery of 15 months (67% survival at 1 year) compared with only 3 months (20% survival at 1 year) when lymph node metastases were present (Crawshaw J et al, 1998).

5.2.3 Muscle Neoplasms

The third most commonly reported intestinal neoplasm in dogs is of smooth muscle lineage (leiomyoma and leiomyosarcoma). A more recently reported variant of leiomyosarcoma and occasionally leiomyoma in dogs is the GI stromal tumor.

Gastrointestinal stromal tumors (GISTs) are well documented in humans and have been also reported in dogs (Gillespie V. et al, 2011). These non-lymphoid tumors of mesenchymal origin were originally diagnosed as leiomyosarcomas, although some were leiomyomas. Histologically, GISTs are highly cellular mesenchymal tumors that do not show ultrastructural characteristics consistent with smooth muscle differentiation. GISTs

are thought to arise from multipotential stem cells phenotypically similar to interstitial cells of Cajal, driven by activating mutations in KIT gene. These cells regulate intestinal motility via an autonomic pacemaker effect. Although they can differentiate into smooth muscle cells if deprived of tyrosine-protein kinase Kit, GISTs are a discrete clinical entity from leiomyosarcoma (Miettinen M et al, 2002). GISTs are distinguished by high vimentin immunoreactivity, low alpha smooth muscle actin reactivity, CD117 (Kit) reactivity, and a site predilection for the large intestine (compared to the stomach for leiomyoma) (Frost D et al, 2003; LaRock RG and Ginn PE, 1997). Activating mutations were identified in KIT exon 11 encoding the juxtamembrane domain in two of four cases examined (Frost D et al, 2003). CD117 reactivity is considered a major diagnostic criteria and in many studies is used to distinguish GISTs from leiomyosarcomas (Russell KN et al, 2007; Maas CPHJ et al, 2007). When stratified as such, 28 of 42 leiomyosarcomas in dogs were reclassified as GISTs and only 2 of the 28 cases of GISTs (7%) gave rise to metastases. Investigators also found that GISTs were significantly more likely to occur in the large intestine, particularly the cecum, and leiomyosarcomas in the stomach and small intestine (Russell KN et al, 2007).

Considering these findings, the incidence of true leiomyosarcoma is likely low because many previously reported cases may have actually been GISTs. Leiomyomas occur more commonly in the stomach but have also been reported in the esophagus, small intestine, and colorectum (Frost D et al, 2003).

Extramedullary plasmacytoma (EMP) refers to solitary tumors with no evidence of systemic multiple myeloma. Case reports of GI EMPs in dogs exist, though uncommon. In one study, a fourth of EMPs were found in the digestive system, particularly in the

mouth (Platz SJ et al, 1999). One case report in a dog with EMP of the colon and rectum was associated with monoclonal gammopathy (Trevor PB et al, 1993).

Finally, one dog was diagnosed with ganglioneuroma of the rectum and experienced long-term survival following surgical resection (Reimer ME et al, 1999).

When tumors of the GI system metastasize, sites of predilection in decreasing frequency include mesenteric lymph nodes (especially adenocarcinoma), liver (especially leiomyosarcoma), mesentery, omentum, spleen, kidney, bone, peritoneum/carcinomatosis, and lung. Interestingly, metastases from intestinal adenocarcinoma were discovered in three dogs initially presented for testicular masses (Crawshaw J et al, 1998; Birchard SJ et al, 1986; Cribb AE, 1988; Prater MR, 2000). One dog was presented for multiple cutaneous masses that suggested round-cell or epithelial malignancy on cytology, for which IHC confirmed an epithelial origin. A primary small intestinal adenocarcinoma with additional visceral metastasis was identified at necropsy (Juopperi TA et al, 2003).

5.3 Cancer of the Gastrointestinal Tract: Molecular Aspects

With an increasing armamentarium of molecular diagnostics, insights into the pathogenesis, progression, and prognosis of tumors are constantly emerging. Cellular adhesion and invasion, such as tenascin-C- (Mukaratirwa S et al, 2003; Mukaratirwa S et al, 2004), versican, hyaluronan (Mukaratirwa S et al, 2004), β -catenin, and e-cadherin (McEntee MF and Brenneman KA, 1999; Restucci B et al, 2009; Aresu L et al, 2010), stromal development and progression of intestinal neoplasia, all play a role in the pathogenesis. The importance of the relationship between a tumor cell and its stroma should not be overlooked. Although molecular markers/targets likely play an important role in

intestinal tumors, the utility of these in diagnosis, prognosis, and therapy in companion animal species, with the exception of GIST and CD117 expression, is limited and further studies are auspicious to clarify their importance and pave the way for a standardized clinical use (Gillespie V et al, 2011).

Measures of cellular proliferation include markers such as argyrophilic nucleolar organizer regions (AgNORs). COX enzymes are responsible for prostaglandin synthesis, and COX-2 is overexpressed in many head/neck and genitourinary tumors, creating a possible therapeutic target. COX-2 has been identified in both benign and malignant small intestinal and colorectal epithelial tumors in dogs, although the number of positive cells varies and in some studies was very low (Knottenbelt C et al, 2006; McEntee MF et al, 2002). The value of COX inhibitors in the treatment of intestinal tumors is therefore disputed.

6.LYMPHOMA

6.1 classification system

Generally speaking lymphomas arise from clonal expansion of lymphoid cells with distinctive morphologic and immunophenotypic features. Many histologic systems have been used to classify non-Hodgkin lymphomas (NHLs) in humans, and some of these have been applied to lymphomas in dogs and other species. The NCI-Working Formulation (the National Cancer Institute of the US proposed classification of non-Hodgkin's lymphomas in 1982) and the updated Kiel system (Lennert K and Feller A, 1990) have been adapted to canine tumors with some success. The World Health Organization (WHO) also publishes a histologic classification scheme, which uses the revised European American lymphoma (REAL) system as a basis for defining histologic categories of hematopoietic and lymphoid tumors in domestic animals (Valli VE et al, 2002). This system incorporates anatomic, histologic, and immunophenotypic criteria (B- and T-cell immunophenotype), with the goal of enabling an accurate and reproducible diagnosis of specific neoplastic entities. This should theoretically assist in better tailoring treatment protocols and correlation with prognosis, besides an improved comparability of doctors' findings.

• **TABLE 32-1** World Health Organization Classification System for Canine Lymphoma

CATEGORY	PERCENTAGE		
	SUEIRO ET AL (N = 55)	VEZZALI ET AL (N = 123)	UNIVERSITY OF WISCONSIN (N = 122)
B-cell neoplasms	72.7	78.9	59.0
Precursor B lymphoblastic leukemia/lymphoma	—	2.4	8.2
B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma	1.8	2.4	0.8
Lymphocytic lymphoma—intermediate type	—	0.8	—
Lymphoplasmacytic lymphoma	10.9	3.3	0.8
Mantle cell lymphoma	—	1.6	—
Follicular center cell lymphomas	1.8	2.4	—
Marginal zone lymphoma (splenic, nodal, mucosa-associated lymphoid tissue)	—	3.3	2.5
Plasma cell myeloma/plasmacytoma	—	16.3	9.8
Diffuse large cell lymphoma	56.3	33.3	24.6
T-cell-rich, B-cell lymphoma	—	0.8	—
Large cell immunoblastic lymphoma	—	10.6	10.7
Mediastinal (thymic) large B-cell lymphoma	—	0.8	—
Burkitt's lymphoma/leukemia	—	0.8	1.6
T-cell and natural killer (NK) cell lymphomas	21.8, 5.4*	21.1	41.0
Precursor T lymphoblastic lymphoma/leukemia	—	6.5	9.8
T-cell chronic lymphocytic leukemia (CLL)	—	3.3	0.8
Intestinal T-cell lymphoma	—	4.1	4.1
Mycosis fungoides/Sézary syndrome	—	1.6	11.5
Cutaneous nonepitheliotropic lymphoma	12.7 [†]	3.3	—
Anaplastic large cell lymphoma	1.8	—	0.8
Peripheral T-cell lymphoma, unspecified	20.0	2.4	13.1 [‡]

*Non-B, non-T lymphomas.

[†]Three B-cell and four T-cell cutaneous lymphomas, not specified as epitheliotropic/nonepitheliotropic.

[‡]Includes one T-zone lymphoma.

Modified from Sueiro FAR, Alessi AC, Vassallo J: Canine lymphomas: A morphological and immunohistochemical study of 55 cases, with observations on p53 immunoprotein expression, *J Comp Pathol* 131:207–213, 2004; and Vezzali E, Parodi AI, Marcato PS, et al: Histopathological classification of 171 cases of canine and feline non-Hodgkin lymphoma according to the WHO, *Vet Comp Oncol* 8:36–49, 2009.

Box 32-1 World Health Organization's Clinical Staging System for Lymphoma in Domestic Animals

Anatomic Site

- A Generalized
- B Alimentary
- C Thymic
- D Skin
- E Leukemia (true)*
- F Others (including solitary renal)

Stage (to include anatomic site)

- I Involvement limited to a single node or lymphoid tissue in a single organ.[†]
- II Involvement of many lymph nodes in a regional area (±tonsils).
- III Generalized lymph node involvement.
- IV Liver and/or spleen involvement (±stage III).
- V Manifestation in the blood and involvement of bone marrow and/or other organ systems (±stage I-IV).

Each stage is subclassified into:

- a Without systemic signs
- b With systemic signs

*Only blood and bone marrow involved.

[†]Excluding bone marrow.

Table 32-1 shows some of the WHO categories in three different surveys, including a 2-year survey (2008-2009) of canine necropsy and biopsy cases at the University of Wisconsin-Madison Veterinary Medical Teaching Hospital pathology service (Sueiro FA et al, 2004; Vezzali E et al, 2009); some of the less common categories in the WHO system were not represented and are not listed. The WHO system is mainly focused of histopathologic aspects and provides accurate and consistent reproducible diagnostic results similar to the system used in human pathology; accuracy among a group of pathologists examining 300 cases was at a 83% agreement score, and accuracy in evaluating the six most common diagnoses (80% of the cases) was 87% (Valli VE et al, 2011). However, there is the need of further clinical studies in order to correlate the various categories of disease with biologic behaviour, response to treatment, and prognosis. Preliminary results indicate dogs with indolent lymphoma (e.g., marginal zone lymphoma, follicular lymphoma, B- or T-cell small cell lymphoma, T-cell-rich B-cell lymphoma, and T-zone lymphoma) maintain normal activity and appetite levels even during advanced stages of disease and experience long-term survival even with limited or no therapy (Stefanello D et al, 2011; Valli VE et al, 2006; Flood-Knapik KE et al 2012). The NCI-WF was developed to allow investigators to “translate” among the numerous classification systems so that clinical trials could be compared in humans. Most of the studies agree that canine lymphomas tend to be to a great extent intermediate or high grade; however, diffuse immunoblastic forms appear to predominate in the United States, whereas the follicular large cell variant is prevalent in Europe. A comparison of European and American classifications is warranted based on this discrepancy.

The WF categorizes tumors according to pattern (diffuse or follicular) and cell type (e.g., small cleaved cell, large cell, immunoblastic), but it does not include information about the immunophenotype of the tumor. The WF subtypes correlate with the biology of the tumor and the patient survival time. The updated Kiel classification includes the architectural pattern, morphology (centroblastic, centrocytic, or immunoblastic), and immunophenotype (B-cell or T-cell) of the tumor cells (Lennert K et al, 1990). In both systems, tumors can then be classified as low-grade, intermediate grade, or high grade malignancies. Low-grade lymphomas composed of small cells with a low mitotic rate typically progress slowly and are associated with long survival times but are ultimately incurable. High grade lymphomas with a high mitotic rate progress rapidly but are more likely to respond initially to chemotherapy and, in humans, are potentially curable.

Several features of canine lymphomas become apparent when these classification systems are applied. The most striking difference between canine and human lymphomas is the scarcity of follicular lymphomas in the dog (Sueiro FA et al, 2004; Vezzali E et al, 2009). Some diffuse lymphomas in the dog may initially be follicular, but these may progress to the more aggressive, diffuse form by the time of diagnostic biopsy. The most common form of canine lymphoma is diffuse large-cell lymphoma, a high grade tumor most commonly of B-cell origin (Vezzali E et al, 2009; Valli VE et al, 2011; Carter RF et al, 1986). Only a small percentage of canine lymphomas (5.3% to 29%) are considered low-grade tumors. High-grade lymphomas occur frequently if diffuse large-cell lymphomas, classified as intermediate grade in the WF, are considered high-grade, as in the updated Kiel classification (in which they are labeled as diffuse centroblastic lymphomas). A documented difference exists in the prevalence of the various immunopheno-

types based on breed (Modiano JF et al, 2005). For example, Cocker Spaniels and Doberman Pinschers are more likely to develop a B-cell lymphoma, Boxers are more likely to have T-cell lymphoma, and Golden Retrievers appear to have an equal likelihood of B- and T-cell tumors. To be clinically useful, these classification systems must eventually yield information about response to therapy, maintenance of remission, and survival. Some studies suggest that the subtypes in the WF can be correlated with survival, and the Kiel system may be useful for predicting relapse (Teske E et al, 1994; Ponce F et al, 2004). In most studies, high-grade lymphomas achieve a complete response (CR) to chemotherapy significantly more often than low-grade tumors. However, dogs with low-grade tumors may live a long time without aggressive chemotherapy (Valli VE et al, 2006; Flood-Knapik KE et al, 2012). Dogs with T-cell lymphomas (with the exception of low grade T-cell subtypes) have shown a lower rate of CR to chemotherapy and shorter remission and survival times than dogs with B-cell tumors (Vail DM et al, 1996; Ruslander DA et al, 1997; Teske E, van et al, 1994; Appelbaum FR et al, 1984). Furthermore, T-cell lymphomas tend to be associated with hypercalcemia (Rosol TJ, Capen CC, 1992; Weir EC et al, 1988), which can lead to severe complications.

In the veterinary literature, 60% to 80% of canine lymphomas are of B-cell origin; T-cell lymphomas account for 10% to 38%; mixed B- and T-cell lymphomas account for as many as 22%; and null-cell tumors (i.e., neither B-cell nor T-cell immunoreactive) represent fewer than 5%. The development of monoclonal antibodies to detect specific markers on canine lymphocytes has made immunophenotyping of tumors in dogs routinely available in many commercial laboratories. Such techniques can be performed on paraffin-embedded samples, from tissue microarrays, on cytologic specimens obtained by

fine-needle aspiration (FNA) of lesions, or by flow cytometric analysis of cellular fluid samples (e.g., peripheral blood, effusions) and lesion aspirates.

The Rappaport classification system, proposed in 1956 for human NHLs, describes the architectural pattern (follicular or diffuse) and the cytologic features (well differentiated, poorly differentiated, or histiocytic) of lymphomas (Rappaport H et al, 1956; Rappaport H, 1966).

This system has not proved useful in providing prognostic information or in driving therapy in dogs with lymphoma because of the low number of follicular lymphomas in dogs, the problematic “histiocytic” subgroup, and the failure to account for different morphologic and immunologic cell types. One criticism of the Rappaport, Kiel, and WF classification systems is that they fail to include extranodal lymphomas as a separate category. The WHO system includes anatomic location as a factor in determining certain categories. Although differences between nodal and extranodal tumors in biologic behaviour and prognosis are well recognized, comparative information about the histogenesis of these tumors is lacking. For example, in humans small-cell lymphomas arising from mucosa-associated lymphoid tissue (MALT) are composed of cells with a different immunophenotype than that of other small-cell lymphomas (i.e., MALT lymphomas typically are negative for both CD5 and CD10). With the exclusion of cutaneous lymphoid neoplasms, a detailed characterization of extranodal lymphomas in dogs has yet to be achieved. Although cutaneous lymphoma is a heterogeneous group of neoplasms that includes an epitheliotropic form resembling mycosis fungoides and a non-epitheliotropic form, most cutaneous lymphomas have a T-cell phenotype (Fry MM et al, 2003; Day MJ, 1995). To conclude, it is important to determine the histologic grade of canine lym-

phomas as low (small lymphocytic or centrocytic lymphomas) or intermediate to high (diffuse large cell, centroblastic, and immunoblastic lymphomas) and the architecture as diffuse or follicular. Furthermore, determining the immunophenotype of the tumor provides useful information. Response rates to chemotherapy are, in general, better in animals with B-cell tumors and intermediate- to high-grade lymphomas. Dogs with low-grade lymphomas can have a long survival expectancy even without aggressive therapy.

6.1.1 Intestinal lymphoma

Among the intestinal tumors of hematopoietic cell origin, lymphoma is the most common in dogs (Guiford WG et al, 1996), with primary intestinal lymphoma being less common than the multicentric form (Head et al, 2002). Although it was previously thought that primary intestinal lymphomas in dogs were of B-cell origin, recent reports have indicated that the majority of canine intestinal lymphoma are of T-cell origin (Coyle KA et al, 2004; French RA et al, 1996; Steinberg H et al, 1995). Of 49 dogs with intestinal lymphoma, 38 cases were of T-cell phenotype with epitheliotropism, three cases were nonepitheliotropic lymphoma of B-cell origin, and eight cases could not be immunophenotyped. T-cell lymphomas of intestinal origin have been divided into two types based on the morphological characteristics of the tumor cells: small- to moderate-sized lymphoma showing epitheliotropic behavior, and moderate- to large-sized lymphoma with or without epitheliotropism. The former type has been classified as intestinal T-cell lymphoma according to the World Health Organization (WHO) classification system (Valli VE et al, 2002) and is similar in cell morphology, immunophenotype, and epitheli-

otropism to previously reported cases (Coyle KA et al, 2004; French RA et al, 1996; Steinberg H et al, 1995). Epitheliotropic T-cell lymphoma of the intestinal tract, also known as intestinal T-cell lymphoma, has been described as a slowly progressive small cell lymphoma of the enteric tract, which appears to arise from a background of chronic inflammatory bowel disease (French RA et al, 1996).

K. Ozaki et al (2006) described mast cell tumors of the canine intestinal tract. However, human intestinal T-cell lymphomas, especially the pleomorphic moderate and large cell types, are usually accompanied by eosinophil infiltration, and eosinophil infiltration has been observed in peripheral/extranodal T-cell lymphomas in animals. These morphological characteristics were very similar to those of moderate- to large-sized T-cell lymphoma of intestinal origin. These results suggest that, in dogs, T-cell lymphomas of intestinal origin resemble mast cell tumors of intestinal origin with respect to cell structure and eosinophil infiltration. Canine intestinal T-cell lymphoma without epitheliotropism has been reported (Ozaki K et al, 2006). Alimentary lymphoma of dogs is accompanied by lymphoplasmacytic infiltration (Couto et al, 1989) An epitheliotropic alimentary form of canine T-cell lymphoma has also been found to be concomitant with lymphocytic-plasmacytic enteritis (French RA et al, 1996). Alimentary lymphoma has aggressive behavior and a poor prognosis (Couto CG et al, 1989). Epitheliotropism was not related to prognosis. Canine T-cell lymphomas, characterized by small to large cell types and eosinophil infiltration, developed in the small intestine. Diagnostic pathologists should be aware of T-cell lymphoma as a differential diagnosis for intestinal round cell tumors with eosinophilic infiltrates. Since these tumor cells were very similar to mast cell tumors of

gastrointestinal origin, immunostaining for mast cell and lymphocyte markers was required for definitive diagnosis.

7. DIAGNOSTIC PROCEDURES

7.1 History and Clinical Signs

Dogs with GI or alimentary lymphoma are usually presented with aspecific GI signs.

The duration of the preclinical stage typically averages 6 to 8 weeks but can range from less than 1 day to several months (Berrocal A et al, 1989; Bennett PF, et al, 2002; Williams LE et al, 2003).

Clinical signs include: weight loss, diarrhea, vomiting, and anorexia and less frequently melena, anemia, and hypoglycemia (mainly if smooth muscle tumors).

Clinical signs often relate to location of the tumor within the GI tract. Proximal lesions more commonly result in vomiting, small intestinal lesions in weight loss, and large bowel lesions in hematochezia and tenesmus (Gröne A et al, 1994; Hause WR et al, 1981). Dogs may present clinical signs relating to intestinal obstruction, such as anorexia, weight loss, and vomiting. Although uncommon, perforation and septic peritonitis can occur (Messinger JS et al. 2009). Smooth muscle tumors are located within the muscular layer of the intestines and not within the lumen and evidence of GI bleeding is often absent, but anemia and melena have been reported (Williams LE et al, 2003).

Canine lymphoma also may be associated with paraneoplastic syndromes (PNSs), which are neoplasm-associated alterations in bodily structure and/or function that occur distant to the tumor. They are a diverse group of clinical aberrations that are associated with the non-invasive actions of the tumor. Often the PNS parallels the underlying malignancy, therefore a successful treatment of the tumor leads to disappearance of the PNS. Alternatively, recurrence of the PNS after an apparent successful treatment sig-

nals a tumor recurrence and often significantly precedes a clinical detection of the tumor.

PNSs are often the first sign of malignancy, and the PNS may be a hallmark of a specific tumor histotypes. Therefore, an understanding and appreciation for the types and underlying causes of these syndromes are paramount for early cancer detection and appropriate therapy. In addition, a PNS may result in greater morbidity than that associated with the actual tumor. The causes of PNSs are quite variable; they are usually caused by the production of small molecules (e.g., hormones, cytokines, or peptides) that are released into the circulation to cause effects at distant sites or by immune cross-reactivity between malignant and normal tissues. Some PNSs are due to functional mutations that result in over-expression of the small molecules in question, whereas many non-endocrine PNSs are of unknown etiology. PNSs are recognized commonly in both human and companion animal cancer patients

7.1.1 Physical examination

An abdominal mass may be palpated on initial examination in approximately 20% to 40% of dogs with lymphoma (Couto CG, et al, 1989) and 20% to 50% of dogs with non-lymphomatous solid intestinal tumors (Crawshaw J, et al, 1998; Paoloni MC, et al, 2002; Birchard SJ et al 1986).

Pain and fever were reported in 20% of dogs with lymphoma in one study (Couto CG, et al, 1989). Digital rectal examination may identify masses or annular strictures due to rectal tumors or polyps in as high as 63% of dogs (Paoloni MC, et al., 2002; Church EM et al 1987; Kosovsky JE, et al, 1992)

7.2 Clinical pathology

7.2.1 Complete Blood Count

Anemia is common in dogs with intestinal tumors and is often not characterized but may occur in conjunction with melena and elevated blood urea nitrogen (BUN). In most studies, anemia is present in nearly 40 % of affected dogs. Leukogram changes are also common, including leukocytosis in 25 to 70 percent of dogs (Crawshaw J, et al, 1998; Kapatkin AS, et al, 1992; Carreras JK, et al, 2003; Kosovsky JE, et al 1988). Left shift as well as monocytosis may be observed in some patients (Birchard SJ et al 1986).

7.2.2 Chemistry Profile

Biochemical abnormalities between dogs with different intestinal tumors are similar. As a result of malabsorption, hypoproteinemia may be present in one-fourth to one-third of patients. In a study by Price *et al* (1991) hypoalbuminemic dogs being treated for multicentric lymphoma had 50 percent shorter remission times than dogs that were presented with normal albumin levels. Albumin plays an important role in physiological function, including drug binding and transport, as well as maintenance of vascular oncotic pressure (Guilford et al, 1996; Hughes D., 2000). It works also as an antioxidant, i.e. a scavenger of free radicals, which are proved to play a role in the pathogenesis of neoplastic diseases (Halliwell B et al, 1990). Although a trend has been observed between survival rates and increased albumin level at presentation, no significant correlation has been

demonstrated; the effect of low albumin on survival time for gastrointestinal lymphoma remains unclear.

Other common abnormalities include elevated liver enzymes, specifically alkaline phosphatase in 15% to 33% of dogs (Crawshaw J et al, 1998; Kapatkin AS, et al, 1992; Paoloni MC, 2002; Birchard SJ, 1986; Carreras JK, et al, 2003; Kosovsky JE, et al 1988). In a study, Joseph David Frank and colleagues (2007) found that a significant portion of examined dogs had hypocalcemia at presentation, although in all but one of these dogs calcemia was normal if corrected for hypoalbuminemia, making this finding of little clinical significance.

7.2.3 Anatomopathological Perspectives of the Gastrointestinal Tract

7.2.3.1 Cytology

As with other anatomic sites, cytology of the intestinal tract can help to differentiate among major tumor types. Cytologic evaluation plays several important roles in veterinary oncology that aid in clinical decision making, including making a tentative or definitive diagnosis, planning diagnostic and treatment strategies, determining prognosis through staging, detecting recurrence, and monitoring response to therapy. An understanding of the advantages and limitations of cytologic evaluation is necessary in order to effectively apply this diagnostic modality in clinical oncology.

Advantages of cytologic evaluation include the ability to study the morphologic appearance of individual cells, the relatively low risk of this procedure to the animal patient and the lower cost compared with biopsy, and the promptness of results.

Cytologic evaluation has nonetheless several limitations. The amount of tissue sampled is small compared with that obtained from a biopsy, resulting in cytologic samples possibly not fully representative of the lesion. Samples quality may be poor because of factors intrinsic to the lesion or poor collection technique. Importantly, the inability to evaluate architectural relationships among cells in cytologic samples gives rise to difficulties in differentiating between reactive and neoplastic processes. Examination of histologic samples, in which tissue architecture is preserved, may be required to make a definitive diagnosis of neoplasia, determine tumor type, and assess the extent of the lesion, including metastasis. Even then, some ancillary tests like immunohistochemical staining or tests for clonality may be required.

Often cytologic evaluation precedes a biopsy and provides information that assists in formulating subsequent diagnostic and treatment options.

Some tumors, such as lymphomas, may be diagnosed using exclusively a cytologic evaluation, and treatment can be initiated without the need to collect a histologic sample. For other tumors, such as well-differentiated hepatocellular carcinomas, a cytologic examination permits to narrow the spectrum of differential diagnoses, making it possible for the histologic evaluation to provide a definitive diagnosis. Cytology can often help to classify a tumor as epithelial, mesenchymal, or discrete round-cell; this may be sufficient for a preliminary discussion with the owner about diagnosis and prognosis. Staging the malignancy, monitoring therapy, and detecting recurrence using a cytologic evaluation is more easily accomplished once a definitive diagnosis has been made and cytomorphologic features of the tumor have been described.

An endoscopic biopsy of the duodenal mucosa has been an important tool in the diagnosis of chronic diseases of the small intestinal tract in dogs and cats for at least 10 years. However, obtaining a tissue sample may sometimes be challenging, as evidenced by the sheer number of techniques that have been reported for endoscopic biopsies. In addition, various types of flexible biopsy forceps are available, and the size and depth of tissue sample that are obtained depend on the characteristics of the forceps used. Therefore, it is not surprising that the quality of tissue sample obtained endoscopically can vary widely, and this variation has led to concerns that endoscopically obtained tissue samples can be often inadequate for a diagnosis. Infact, inadequate biopsy samples have been cited as a cause of erroneous diagnoses in veterinary medicine. This concern about the quality of endoscopically obtained tissue samples seems particularly important for the duodenal tract. Firstly, because of the nature of the duodenal mucosa, good quality duodenal samples seem to be relatively harder to obtain as compared with other areas of the alimentary tract and more prone to handle artifacts than do gastric and colonic mucosa samples. Secondly, upper gastrointestinal tract disease causing vomiting or diarrhea often involves the duodenal mucosa, in some cases even more than the gastric mucosa, making it important for a correct diagnosis an excellent sample of duodenal mucosa.

7.2.3.2 Cytology of Lymphoma

Small, well-differentiated lymphocytes measuring 1 to 1.5 times the diameter of an erythrocyte in the dog compose approximately 90% of the population in lymph nodes. The chromatin of these cells is densely clumped with no visible nucleoli. Cytoplasm is scant. These cells are the darkest staining of all lymphocytes. Medium and large lymphocytes, whose nuclei measure 2 to 3 times the erythrocytic diameter, are usually present in low numbers (5% to 10%).

Their nuclei have a fine, diffuse, and light chromatin pattern. Nucleoli may be prominent. The cytoplasm is more abundant and often basophilic. Mature plasma cells represent a small portion of the cells found. Their chromatin is densely clumped and often the nucleus is eccentrically placed within the abundant, deeply basophilic cytoplasm. A pale area or halo is seen adjacent to the nucleus, which indicates the Golgi zone. Occasional macrophages (histiocytes) appear as large mononuclear cells with abundant light cytoplasm, often containing cellular debris. Nuclear chromatin is finely stippled and nucleoli may be found in activated macrophages. Mast cells and neutrophils also may be present in low numbers.

Inflammatory cells are categorized according to the grading system, with a grade of 2 or more indicating the corresponding degree of significant inflammation. The grading system is used to express the presence and magnitude of the neutrophilic or lymphocytic-plasmacytic inflammatory constituents. Severe lymphocytic enteritis may be difficult to differentiate from malignant lymphoma when medium to large lymphocytes are prominent. A comparative correlation with the histologic findings is mandatory.

Cytologic Protocol and Terms Used to Evaluate Lymphoma Cases (Teske et al, 1994).

Determine the cell size based on comparison of the nucleus to the size of an erythrocyte.

- Small: 1-1.5 \times RBC
- Medium: 2-2.5 \times RBC
- Large: $\geq 3 \times$ RBC

Determine the shape of the nucleus and its placement within the cytoplasm.

- Round: Circular with no indentations
- Irregularly round: Few indentations or convolutions
- Convolutated: Several deep indentations
- Clefted: Single deep indentation
- Central vs. eccentric placement

Determine the number, size, visibility, and location of nucleoli within the neoplastic lymphocytes.

- Single vs. multiple
- Large vs. small
- Indistinct: Not visible or barely perceivable
- Prominent: Easily visible
- Central vs. marginal or peripheral placement

Describe the cytoplasm by amount and color. Be sure to note presence of Golgi zone or granulation.

- Scant: Small rim around nucleus
- Moderate size: Amount intermediate between scant and abundant
- Abundant: Nearly twice the size of the nucleus
- Pale: Light basophilia or clear
- Moderate basophilia: Color intermediate between pale and dark blue
- Deep basophilia: Royal blue or darker

Estimate the mitotic index by looking at 5 cellular fields under 40 mm or 50 mm objectives.

- Low: 0-1 mitotic figures per 5 fields
- Moderate: 2-3 mitotic figures per 5 fields
- High: 3 mitotic figures per 5 fields

Tumor grade is morphologically based on cell size and mitotic index.

- Low grade: Low mitotic index and small cell size
- High grade: Moderate or high mitotic index and medium or large cell size

Lymphoma comprises many variants. Definitive diagnosis of lymphoma based on cytologic examination is often possible; however, in some types of lymphoma or in certain discrete round-cell neoplasms, it is the homogeneity of the population, rather than its morphology, that suggests a neoplastic process. In lymphoid organs or other tissues in which there is a reactive or polyclonal infiltrate of lymphocytes, small lymphocytes

should make up more than 50 percent of the lymphoid cells, even as the proportion of large and intermediate lymphocytes increases and plasma cells and other inflammatory cells are found in these reactive lesions. As the proportion of intermediate and large lymphocytes approaches or exceeds 50%, it becomes more difficult to differentiate between a reactive and neoplastic process; this is especially true for the spleen and certain lymph nodes, such as mandibular and mesenteric nodes, that are continuously exposed to antigens. Because of this, sampling of other nodes or tissues is preferred. In addition, cats can show strong lymphocytic responses that can cytologically resemble a lymphoma. In contrast, there are certain types of lymphoma, such as T-cell-rich B-cell lymphomas and Hodgkin's-like lymphoma, that contains a mixture of clonal (neoplastic) and polyclonal (non-neoplastic) populations of lymphocytes. When a diagnosis of lymphoma is not definitive from the cytologic specimen, additional procedures should be carried out, including biopsy with histologic evaluation, immunophenotyping, assessment of clonality, or a combination of these.

Lymphoma can be easily diagnosed cytologically when large or intermediate lymphocytes comprise the majority of the nodal population. Large and medium-sized lymphocytes are defined as those larger than or the same size as a neutrophil, respectively, or that are greater than two times or one-and-a-half to two times the diameter of an erythrocyte, respectively. Cytologic types include immunoblastic or centroblastic types, composed of large cells with visible nucleoli and deeply basophilic cytoplasm, and types composed of medium-sized cells often having indistinct nucleoli. Mitotic figures and tingible-body macrophages, which are macrophages containing nuclear debris from tumor cells, may also be increased, but this is not a defining characteristic.

Cytologic diagnosis of small cell lymphoma is more challenging, especially in tissues such as lymph nodes and spleen with a resident population of small lymphocytes or in tissues such as liver and small intestine in which lymphocytic inflammation is common. In these cases, additional diagnostic tests are required for confirmation and may include one or more of the following: histologic examination, preferably of a whole node or full-thickness piece of intestine; immunophenotyping by immunocytochemical/histochemical staining or flow cytometry; and polymerase chain reaction (PCR) for antigen receptor rearrangement to detect clonality.

Since lymphocytes are fragile, free nuclei and cytoplasmic fragments are frequently observed in aspirates of lymphoma; however, these features can be found in samples from reactive lymphocytic populations and are not criteria for neoplasia.

Infrequently, neoplastic lymphocytes are highly pleomorphic and exhibit moderate to marked anisocytosis, indented or deeply clefted nuclei, ameboid nuclei, multinuclearity, cytoplasmic vacuoles, and aberrant phagocytic behavior. When present, a few, some, or most of the neoplastic lymphocytes in a given tumor may have these features and may be mistaken for neoplastic histiocytes (Jaffe ES, 2009). Sometimes neoplastic lymphocytes contain fine or coarse pink cytoplasmic granules, suggestive of a T- or NK-cell phenotype. In large granular lymphomas, the lymphocytes contain large, coarse, pink granules and are thought to be cytotoxic T- or NK-cells.

Views differ regarding the accuracy of cytological diagnosis of enteropathy-type lymphomas. In one study done by Bonfanti and colleagues, results of fine-needle biopsy of deep abdominal organs were consistent with the diagnosis of inflammatory versus neoplastic disease in 89% of cases (Bonfanti et al, 2004). However, in another study by the

same authors, cytological examination of gastrointestinal tumors was consistent with histological findings in only 72% of cases (Bonfanti et al, 2006). Although histology is preferred for accurate cell typing and grading, cytological examination has been identified as an accurate means to diagnose canine lymphoma (Vail DM et al, 2001).

7.2.3.3 Histology

Although histological examination of all layers and full thickness biopsies of GI wall remains the gold standard the diagnosis and treatment of gastrointestinal disease in the dog and cat are increasingly based on the collection and interpretation of mucosal biopsy samples obtained endoscopically from one or more gastrointestinal sites. There are many stages in this process in which errors may occur, thereby influencing the clinical outcome. These stages include the endoscopic biopsy procedure, the processing and embedding of the small and fragile tissue samples, and the microscopical interpretation of the tissue changes by the diagnostic pathologist (Willard et al., 2001, 2002). For many clinicians and pathologists, the histopathological interpretation has proved to be the most contentious and frustrating step in the diagnostic sequence. This interpretation may be complicated by inadequacies in the number and quality of the tissue samples, by fragmentation and unfavourable orientation of these samples during processing.

The prognosis of alimentary lymphoma is poor, compared to the multicentric form, regardless of the treatment. Therefore, it is important to make a definitive diagnosis of alimentary lymphoma. In many cases lymphoma is currently diagnosed on the basis of cytological or histopathological findings. However, it is frequently observed that the se-

vere lymphocytic-plasmacytic inflammation that occurs in conjunction with alimentary lymphoma makes it difficult to distinguish lymphocytic-plasmacytic enteritis (LPE) from alimentary lymphoma, especially when the tumor cells are well differentiated (e.g., low grade lymphoma).

Usually, the pathologist diagnoses an alimentary lymphoma in a sample with the following characteristics: atypical lymphoblast infiltration, infiltration of lymphocytes with destruction or replacement of the normal architecture of the epithelial cells layer, lamina propria or crypta. However, a histopathological distinction of an alimentary lymphoma from lymphocytic-plasmacytic enteritis is often difficult.

7.2.3.4 Immunohistochemistry

Immunohistochemical staining is carried out on formalin-fixed, paraffin-embedded tissue or, less commonly, on frozen sections. Immunocytochemistry (ICC) uses the same methods and reagents but on fine-needle aspirates. Antibodies to specific proteins are applied to the tissue, generally followed by a secondary antibody of a different species than the patient or the primary antibody.

The secondary antibodies are conjugated to enzymes that catalyze a color change when a substrate is added. This method allows pathologists to identify individual cells that do or do not express the protein of interest and to put these cells in the context of tissue architecture.

In veterinary oncology, IHC is most commonly used to distinguish sarcomas from carcinomas and to phenotype lymphomas. The first application involves staining tumor sections with antibodies to the cytoskeletal proteins vimentin and cytokeratin.

Vimentin is found in mesenchymal origin tumors (e.g., osteosarcomas, fibrosarcomas). Cytokeratin is expressed by epithelial origin cancers (carcinomas). Although the distinction between these two types of tumors is generally straightforward, it is common to request vimentin/cytokeratin staining when results are not clear.

IHC is particularly important in subclassifying lymphoma. The 2008 World Health Organization (WHO) classification of lymphomas describes more than 40 lymphoproliferative disorders (lymphoma and leukemia) in humans (Jaffe ES, 2009). All subclassifications begin with phenotype (B- versus T-cell). The origin and biologic behavior of different subtypes of lymphoma is different enough that it is not scientifically justified to consider lymphoma as a single disease. Therefore, when conducting therapeutic trials, epidemiologic studies, and any other types of analyses on canine lymphomas, it will be necessary to examine individual subtypes separately.

The antibodies used to determine the B- or T-cell origin of lymphoma in formalin-fixed sections are anti-CD3 (which identifies T-cells) and one of several anti-B-cell antibodies, including CD79a, Pax5, and CD20. All of these antibodies recognize the cytoplasmic portions of the target antigen and the process of fixation permeabilizes cells to allow access of antibodies to these epitopes. The antibodies also recognize conserved sequences of proteins and are therefore useful in a variety of different species. Further sub-classification of lymphoma is possible based on the distribution of neoplastic cells within the lymph node. For example, there is a number of neoplasms of mature B-cells, such as mantle-cell and follicular lymphoma (FL), with very different outcomes. These lymphomas can be differentiated by examining the architecture of a lymph node stained with B-cell antibodies. In human patients, they can also be differentiated by flow cy-

tometry or cytogenetic studies. The human classification has been applied to canine lymphoma by a consortium of pathologists who reached a broad albeit not complete agreement about histologic subtypes (Ponce F et al, 2004).

Unfortunately, the paucity of data on the biologic behaviour of these subtypes in dogs doesn't allow for them to be used in a clinical setting. IHC, however, allows the pathologist to define positive cells into the architecture of the node. Veterinary pathologists recognize many of the WHO categories of lymphoma, including diffuse large B-cell lymphoma, FL, and mantle-cell lymphoma. One study systematically evaluated outcomes using contemporary classifications and found that small cell T-zone lymphoma had the most favorable outcome, whereas Burkitt-like B-cell lymphoma had the worst one (Ponce F et al, 2004). This study, although small, revealed the value of classifying and subclassifying lymphomas using contemporary standards. More recently, an investigation of canine indolent T-cell lymphoma, diagnosed by histology coupled with IHC, revealed that treatment of this form of lymphoma with a CHOP-based protocol resulted in the same outcome as treatment with chlorambucil and prednisone.

This is the first study clearly demonstrating the utility of histology and IHC in guiding treatment decisions (Flood-Knapik KE et al, 2012).

7.2.5 Molecular biology

The complete genetic code, or DNA sequence, is present within every cell in the body. The effective genetic information that uniquely defines each cell type within the body is defined by the genes expressed (transcribed) as mRNA. The expression of mRNA is more responsible for the phenotype of a cancer than the individual genes and mutations harbored by the tumor. When assessing the level of expression of one or a few genes, real time PCR is most commonly used. Assessment of the global level of gene expression is carried out with microarrays and is called *gene expression profiling*.

Both methods measure relative expression of message when compared to a control gene whose expression is thought to be more or less constant in all cells and universal. These methods are both likely to be replaced over the next 10 years with new technologies, which will allow investigators to count the absolute number of genes or transcripts in a sample.

Real-time PCR (also called *Q-PCR*) refers to the quantitative measurement of DNA, either single genes, or more commonly, RNA that has been reverse transcribed to cDNA. The principle of real-time PCR is that DNA is amplified using primers, just as in a routine PCR reaction, but at each round of amplification, fluorescence relative to the amount of PCR product is quantified. Unlike endpoint PCR, in which the reaction runs to completion and the product is separated by size, real-time PCR is highly quantitative.

The two main methods for quantifying DNA in real-time PCR have different advantages and disadvantages. The simplest and least expensive method is the use of the DNA-binding dyes, such as *SYBR green*. This dye fluoresces when it intercalates into DNA.

Therefore, at each round of amplification, more dye is intercalated and the degree of fluorescence increases.

Eventually, the fluorescence reaches saturation, but the endpoint is less important than the log phase of DNA increase. The amount of starting material is compared between samples by determining at which amplification cycle the degree of fluorescence crosses a given threshold. SYBR green will intercalate into any DNA product, so this method is only accurate when the primers are highly specific and amplify only one gene product.

An alternative method uses fluorescently labeled DNA probes. These are short segments of DNA complementary to the target sequence between the two primer sites. A fluorescent molecule attached to the probe is quenched until the 5' to 3' exonuclease activity of the Taq polymerase releases the fluorescent molecule. As the amount of PCR product increases proportional to the starting material, the amount of fluorescence increases. The advantage of the probe method is that the use of three primers, instead of two, provides more specificity to the reaction. In order to detect a product, these three different segments of DNA need to bind to the target sequence in three separate places. The assay may be less robust at low target numbers and is slightly more expensive, but it is also significantly more specific. This assay with its increased specificity was used by Yamazaki and colleagues (2008) to quantify individual neoplastic lymphocytes in a background of heterogeneous lymphocytes and thus quantify minimal residual disease.

Real-time PCR can quickly allow for precise quantification of mRNA levels within samples and has a number of defined clinical applications. It is commonly used in the quantification of aberrant oncogenic fusion products. Promyelocytic leukemia gene–retinoic acid receptor (PML-RAR) fusion transcripts in acute promyelocytic leukemia (APL) are

identified by this method in humans (Gallagher RE et al, 2003) and can be used to define molecular remission in patients with APL. Molecular remissions are defined by the absence not only of a clinically detectable disease but also of any tumor-associated transcript in blood, bone marrow, or lymph node. Achieving a molecular remission in patients with leukemias and lymphoma is a superior measure of prognosis over clinical assessments of remission that are based on clinical examination, imaging techniques, or histologic or cytologic assessments of at-risk tissues.

A report by Kaneko et al. (2009) has suggested the application of molecular-based methods as diagnostic tools for canine alimentary lymphoma; these diagnostic tools are useful for the detection of a latent alimentary lymphoma. In a study of Fukushima H. et al. (2009), the overall sensitivity of the PARR analysis in diagnosing canine alimentary lymphoma was 66%, which was lower than that of other lymphoid malignancies.

7.3 Plain and Contrast Abdominal Radiographs

In dogs with intestinal lymphoma, concurrent enlargement of liver, spleen, and/or mesenteric lymph nodes may be found (Couto CG et al, 1989). Plain abdominal radiographs may reveal an abdominal mass in approximately 40% of both dogs and cats, although some reports are higher for solid tumor types and lower for lymphoma. Intestinal lymphoma may be more difficult to identify on plain radiography because of other organ involvement, peritoneal effusion, or diffuse intestinal lesions. An obstructive pattern may also be seen on plain radiographs, with incidence ranging from 10 to 75 percent. Contrast radiography, although now partially replaced following advances in ultrasound, has often been used to evaluate patients with signs of primary GI disease. Contrast radiog-

raphy can help to identify an obstruction, localize a tumor, and view areas of the GI tract that are difficult to image with ultrasonography because of gas accumulation. Contrast radiographs may reveal filling defects in approximately half the cats and dogs with GI neoplasia (Birchard SJ et al, 1986). In dogs with GI lymphoma, all 12 dogs examined had abnormal contrast series.

7.4 Thoracic Radiographs

Thoracic radiographs are critical to the complete evaluation of the cancer patient. For dogs with non-lymphomatous intestinal tumors, yield is low with very few patients presenting with pulmonary metastasis. This may be due to a bias in reporting, because many reports detail outcome of treatment and patients with metastatic disease may not receive treatment. In fact, many case series report no evidence of metastasis on initial evaluation for solid tumors of the intestine in dogs.

7.5 Abdominal Ultrasound

Ultrasound allows a non-invasive localization of the tumor and identification of other sites of metastasis/involvement. It also can guide needle aspiration or needle biopsy or assist in treatment planning. Ultrasound is a more sensitive diagnostic test than radiographs for identifying a mass (Rivers BJ et al, 1997). Ultrasound is also less time-consuming than contrast radiography, and availability together with improved operators' skills for the former have diminished the need for the latter.

Ultrasound findings in dogs with intestinal neoplasia most consistently include thickening and loss of the normal layers in the bowel wall (Penninck DG et al, 2003).

Degree of thickening, distribution of lesions, and symmetry are also used to help differentiating neoplasia from non-neoplastic disease (Gaschen L, 2011).

Intestinal lymphoma in dogs often results in long segments of involved bowel.

In one study, two-thirds of dogs with intestinal adenocarcinoma had hypoechoic tumors, and most had a decreased motility. Masses averaged 4-cm in diameter with a median wall thickness of 1.2 cm (Patnaik AK et al,1977). Mast cell tumors have an eccentric appearance with alteration, but not loss, of wall layering, commonly involving the muscularis propria. Smooth muscle tumors are characteristically large (median diameter 4.8 cm) and anechoic or hypoechoic. Leiomyomas may have a smooth contour (Myers NC and Penninck DG, 1994). Ultrasound has also proven useful in differentiating neoplastic from non-neoplastic intestinal disease. Dogs with tumors have significantly thicker intestinal walls, and 99 percent have a loss of wall layering, compared to a maintenance of wall layering in 88 percent of dogs with non-neoplastic disease. In fact, dogs with a loss of wall layering are more than 50 times more likely to have a tumor than enteritis. Additionally, dogs with an intestinal wall thicker than 1 cm and those with focal lesions are nearly 4 times and 20 times as likely to have a tumor, respectively. Nevertheless, the diagnosis is not always straightforward: fungal masses, as in pythiosis and histoplasmosis, that can mimic neoplasia and a lymphadenopathy can also be caused by an infectious or inflammatory bowel disease. In general, neoplasia exhibits more dramatic thickening with loss of wall layering and greater lymph nodal enlargement, as well as more frequent focal lesions than non-neoplastic intestinal diseases.

7.6 Endoscopy and Laparoscopy

Minimally invasive methods of collecting tissues to aid in diagnosis are increasingly used. Endoscopic findings in dogs with intestinal lymphoma include an irregular cobblestone or patchy erythematous appearance to the duodenal mucosa and poor distensibility and elasticity of the duodenal wall (Miura T et al, 2004). Significant inter-observer variation may occur in the interpretation of biopsy samples. In one study, blinded pathologists assigned a degree of mucosal cellular infiltrate as severe as neoplasia in five clinically normal research dogs. Inter-observer variation is likely to be more pronounced with small tissue samples and this is a limitation of these less invasive approaches.

7.7 Exploratory Laparotomy

When non-invasive or minimally invasive diagnostics in a dog with persistent signs of GI disease fails, an exploratory laparotomy may be the next step. This procedure allows the direct visualization of all abdominal viscera and the acquisition of full thickness biopsies of all segments of intestines and other viscera. Patients with resectable solid tumors may be both diagnosed and treated in one single procedure with resection and anastomosis. In a series of dogs with GI lymphoma, endoscopic biopsies were sometimes difficult to interpret because of lymphoplasmacytic infiltrate, but biopsies obtained via laparotomy confirmed the diagnosis in all cases undergoing surgery (Couto CG et al, 1989). As a side note, it is important in the clinical practice to avoid the pitfall of automatically considering carcinomatosis as an indication for euthanasia.

8. AIM OF THE WORK

This study was focused on the subtype of intestinal lymphoma defined as epitheliotropic or enteropathy-type lymphoma (Valli, 2007), which is the major differential diagnosis of IBD. In epitheliotropic lymphoma, neoplastic lymphoid cells invade the intestinal mucosa with a diffuse pattern, leading to an impairment of the normal functions of digestion and absorption. In clinical practice, gastro-enteric signs are similar to those present in inflammatory enteropathies. The differential diagnosis between IBD and alimentary lymphoma based on clinical, diagnostic imaging and laboratory findings can be very challenging. Patients with alimentary lymphoma show clinical symptoms and signs that are all comparable to patients with inflammatory enteropathy. The endoscopic examination and biopsy of the alimentary tract is paramount to differentiate between IBD and lymphoma and consequently deliver the appropriate treatment.

In most cases of canine lymphoma, which are high-grade subtypes, the presence of a single population of immature lymphoid allows an unproblematic cytologic diagnosis. However, some subtypes of lymphoma (e.g. small-cell lymphoma, follicular lymphoma, etc) require histopathology for a definitive diagnosis because of the inherent limitations associated with cytology. Additional investigation using lineage-specific antibodies may require immunocytochemistry, immunohistochemistry and flow cytometry to differentiate between B- and T-cell lymphomas and can provide some information regarding prognosis.

In enteropathy-type lymphomas, cytology or histology may fail to confirm the diagnosis and a different approach may be required. Surgical full thickness biopsies via laparato-

my and enterotomy is generally considered the best diagnostic methods. However, this test is rarely accepted by the owner in a clinical setting and endoscopic biopsies are generally considered more feasible. This study is focused on the early identification and diagnosis of enteropathy-type lymphoma in the clinical practice, using an endoscopic approach. We outline some cytological guidelines that can be used to differentiate between IBD and lymphoma on squash-prep endoscopic biopsy. The aim of our study is to evaluate the diagnostic accuracy of cytological results in the differential diagnosis between IBD and lymphoma in dogs on endoscopic biopsies. Since a single gold standard is not available for this scope, the integration of history, physical findings, diagnostic imaging, endoscopic features, intestinal cytology, intestinal histology and immunohistochemistry along with a follow-up of each case, was implemented by an expert in gastroenterology in order to reach a definitive diagnosis. Moreover, although the evaluation of histopathologic results is not within the aims of the present work and I did not personally perform histopathology, we report and discuss here the results obtained from the histopathological evaluation and immunohistochemistry of endoscopic biopsies, in order to verify and compare the diagnostic performance of different techniques.

Based on this approach, I have compared the accuracy between cytology and histology.

I have also evaluated the agreement rate of cytological reports obtained by two different independent reviewers, in order to determine whether the proposed cytological clues are repeatable in a clinical setting by differently experienced readers.

9. MATERIAL AND METHODS USED

Inclusion criteria and tissue samples: the present study included retrospectively dogs that suffered from gastrointestinal symptoms such as diarrhea and vomiting and that had been endoscopically examined by Dr. Enrico Bottero between October 2007 and October 2012. Cases were included only if complete clinical data were available, if cytological samples were available for reviewing and if a histological diagnosis was available for each case.

Two biopsy samples were obtained from each lesion using endoscopic biopsy forceps and then submitted to a cytologic and histologic examination. One of the samples was fixed in 10 percent buffered formalin, routinely processed and histopathologically evaluated. All samples were classified into two groups: lymphoma and enteritis groups. Cases of canine enteropathy-type lymphoma diagnosis were confirmed by clinical aspect, EOG, endoscopic features, cytology, histology and follow up. The cytological samples were independently and blindly reviewed from two clinical pathologists with different experience level, respectively, both not specialised in gastroenteric cytology. Histologic endoscopic biopsy of small intestine from each dog were evaluated blindly by one pathologist, and based on the microscopic appearance of the most severely affected section, a diagnosis of IBD, T-cell lymphoma, or B-cell lymphoma was given following published criteria. Biopsy samples of ileum and jejunum were available from all dogs. No gastric or large intestinal biopsies were examined.

Cytological samples were prepared by a “squash technique” as follows.

A small amount of material was placed on a clean glass slide approximately 1/2 inch (1 cm) from the frosted end (Fig. A,B,C). A second clean glass slide was placed over the

specimen at right angles. The specimen was gently but firmly compressed between the two glass slides and in the same continuous motion the top slide was pulled along the surface of the bottom slide, directing the material away from the frosted end (Fig. D,E). In this way, a multicellular mass was turned into a thin monolayer ideal for maximal flattening of individual cells and stain penetration, optimizing the specimen for the microscopic examination. A properly prepared glass slide is characterized by a feather-shaped (oblong) area with a monolayer end referred to as the “sweet spot”. A common mistake is the initial placement of excess sample on the glass slide, resulting in a thick preparation that is not possible to adequately examine microscopically.

IMAGE A-E. Solid material preparation. **A**, The procedure for making a cytologic preparation from a tissue sample is illustrated. Specimens for cytologic examination were obtained during routine endoscopic examination of the small intestine. **B**, Tissue rolling. Small pieces of tissue that cannot be grasped with a forceps for imprinting can be gently placed on a slide using a 25-gauge needle(**C**). **D E**. A second clean glass slide is placed over the specimen at right angles. The specimen is gently but firmly compressed between the two glass slides and in the same continuous motion the top slide is pulled along the surface of the bottom slide, directing the material away from the frosted end.

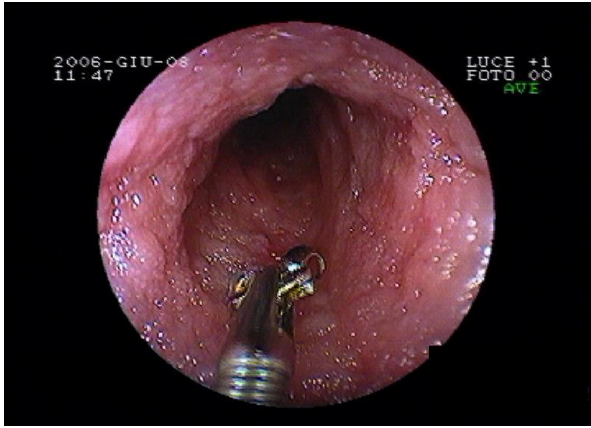


Image A



Image B

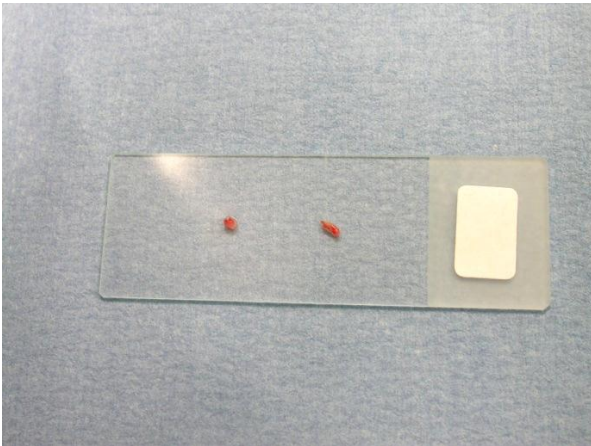


Image C

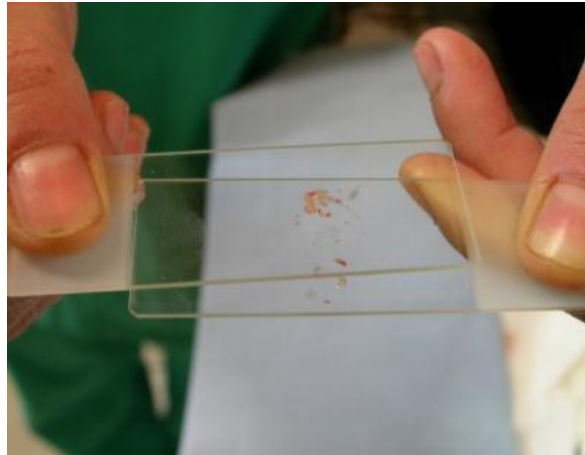


Image D

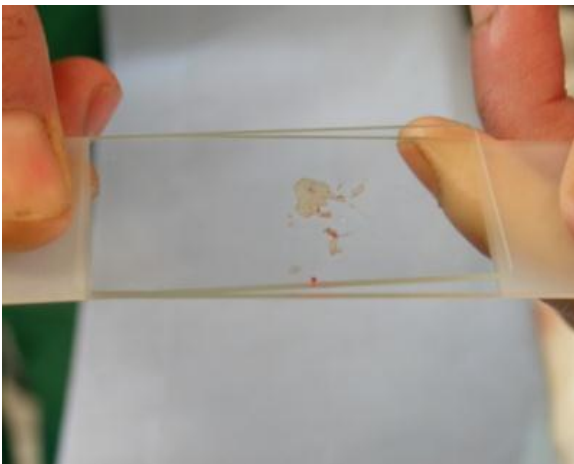


Image E

Staining the specimen: Romanowsky-type stains were utilized because of their rapidity and easiness to use (BOX1). They are a combinations of basic and acidic dyes dissolved in methyl alcohol. These polychromatic stains impart the basophilic and eosinophilic tinctorial properties observed on blood films.

The stained specimen is examined microscopically using the 10 or 20 mm objective for staining quality and uniformity. When a 40 mm objective is to be used, a coverslip is usually placed on the specimen. A temporary mount is made by placing a drop of immersion oil on the specimen, followed by a coverslip. A permanent mount is made with a commercially available coverslip mounting glue (e.g., Eukitt^R, Calibrated Instruments).

BOX 1 Suggested Procedure for Staining Cytologic Sample Using Diff-Quik®Solutions

Fixative: 60 to 120 seconds

Solution 1: 30 to 60 seconds

Solution 2: 5 to 60 seconds†

Rinse under cold tap water: 15 seconds

Examine staining adequacy using low power; eosinophilia or basophilia can be enhanced by returning to Solution 1 or Solution 2, respectively, followed by a rinse.

Air-dry and examine

CYTOLOGY

Description of different cytological features: Cytological smear were evaluated and the following aspects were recorded for each sample:

- ↪ small lymphocytes
- ↪ plasma cells
- ↪ eosinophils
- ↪ immature lymphoid cells
- ↪ membrane alterations of the lymphoid cells
- ↪ atypical mitoses
- ↪ presence of lympho glandular bodies
- ↪ cytomorphologic anomalies in enterocytes

In the second part of the study we have blindly re-evaluated the same samples using a reduced number of selected cytomorphological features. In particular, three cytological features selected as recurrent in the first step of the study were identified independently by two readers:

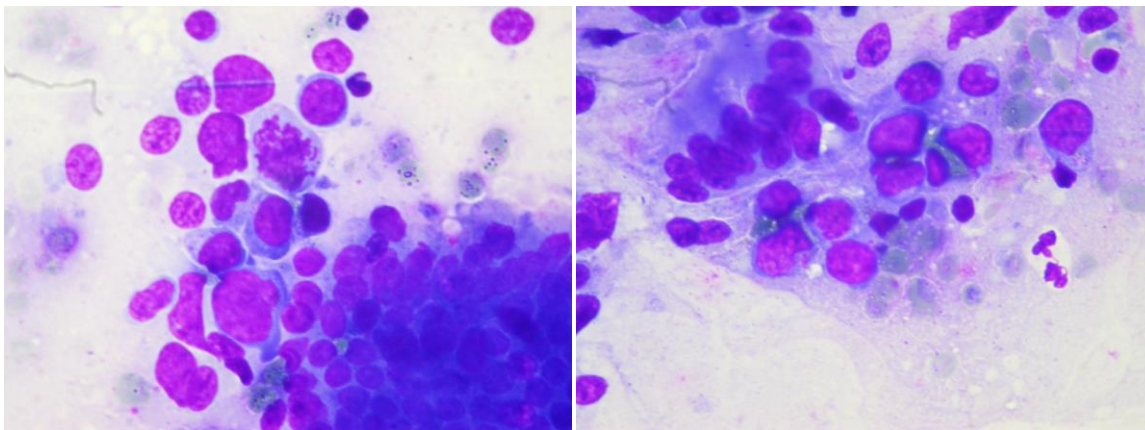
- ↪ presence of lymphoid blast cells
- ↪ fine structural change of the lymphoid blasts's membrane
- ↪ presence of lympho-glandular bodies

For each cytologic feature and reader, sensibility and specificity were evaluated. As reference the final diagnosis drawn by the specialist in gastroenterology who was the only aware of all the results of different diagnostic tests (including histopathology), clinical features, endoscopic aspect and follow up/response to therapy. In order to compare the results obtained by the different readers and the different techniques further evaluation were then performed using the test of Cohen's kappa coefficient for inter-rater agreement. Predictive positive and negative values were also evaluated.

LARGE IMMATURE LYMPHOID CELLS

Medium-sized or large lymphocytes often compose 50% of the total cells in lymphomas. An exception is the presentation of alimentary lymphoma in which reactive lymphocytes represent the majority of the cell population. The population is often homogenous when cell populations are mixed, including different cell sizes present such as small and large lymphocytes, the diagnosis of lymphoma requires additional procedures. The cell size based on comparison of the nucleus to the size of an erythrocyte.

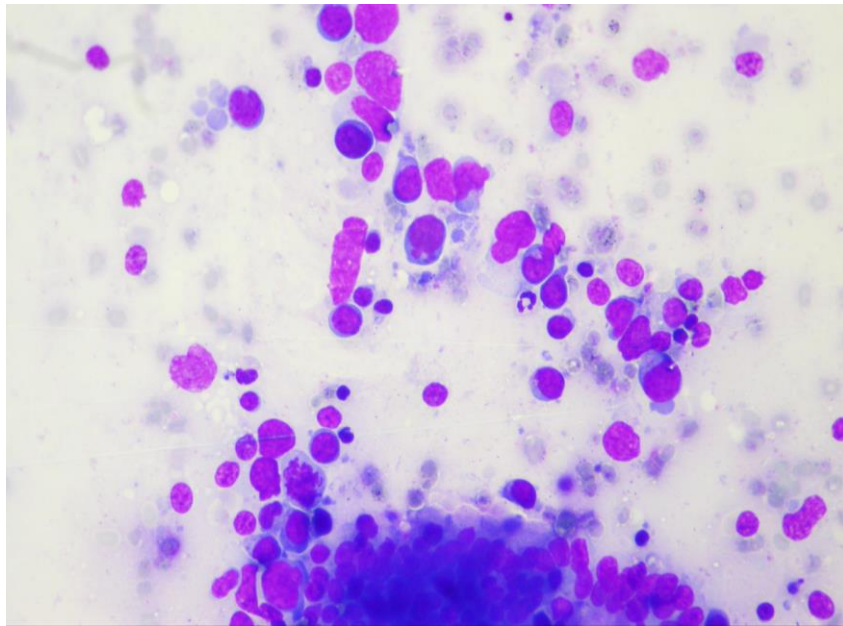
- Small: 1-1.5 RBC
- Medium: 2-2.5 RBC
- Large: >3 RBC



Immature lymphoid cells, intestine,dog. Lymphoma T. (Wright-Giemsa; HP oil)

LYMPHOGLANDULAR BODIES

Lymphoglandular bodies result from the rupture of lymphocytes and appear as small platelet-sized basophilic cytoplasmic fragments. Although they may be seen in benign lymph node conditions, a higher frequency is expected in lymphoma because of the immaturity and fragility of these cells. Lysed nuclei may appear as a lacy, amorphous eosinophilic material.



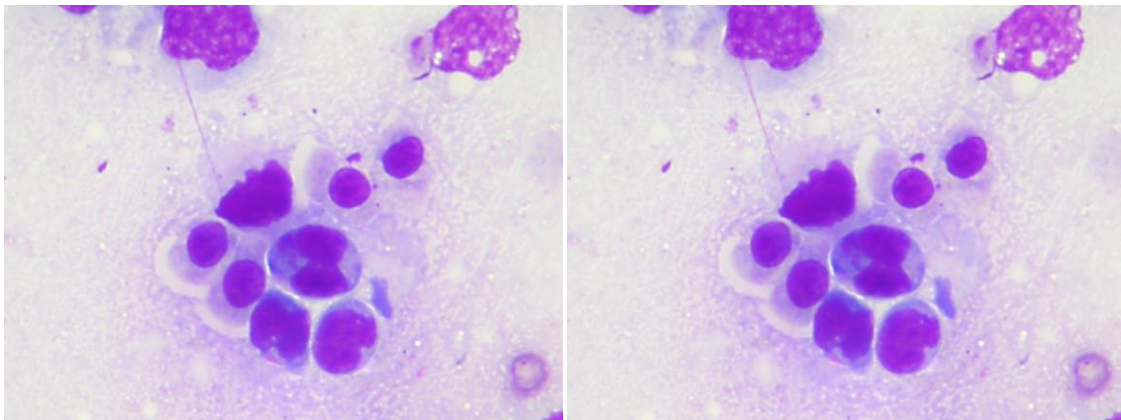
Lymphoglandular bodies, intestine,dog. Prominent basophilic round structures of variable size indicate fragmentation of the cytoplasm. This appearance is often associated with lymphoma but may be found in other conditions having fragile cells. (Wright-Giemsa; HP oil.)

NUCLEAR MEMBRANE

Morphologic appearance of the neoplastic cells has been used along with immunophenotype to further classify the lymphomas for prognostic value (Ponce et al.,2004).

Nuclei are generally described according to different aspects:

- Round: circular with no indentations
- Irregularly round: few indentations or convolutions
- Convolutated: several deep indentations
- Clefted: single deep indentation
- Central vs. eccentric placement



Irregual morphologic appearance of balt membrane, intestine,dog. Lymphoma T.

(Wright-Giemsa; HP oil)

HYSTOPATHOLOGY

Histopatologic evaluation: biopsy samples of ileum and jejunum were available from all dogs. No gastric or large intestine biopsies were examined. The endoscopic tissues consisted of mucosa and submucosa. For all cases, sufficient formalin-fixed, paraffin-embedded samples were available for further testing. Serial sections from paraffin tissue blocks from each of the 20 dogs were cut for routine HE staining.

The canine IBD activity index (CIBDAI) was used for evaluation of the severity of illness (box2)

Box2: Histological Scoring System used to evaluate the degree of intestinal inflammation

Intestinal Histologic Scoring System		
Feature Scored	Score	Description
Inflammation Severity	0	None
	1	Mild
	2	Moderate
	3	Severe
Inflammation Extent	0	None
	1	Mucosa
	2	Mucosa and Submucosa
	3	Transmural
Crypt Damage	0	None
	1	1/3 of crypt damaged
	2	2/3 of crypt damaged
	3	Crypts lost, surface epithelium present
	4	Crypts and surface epithelium lost
Percent involvement	0	0%
	1	1–25%
	2	26–50%
	3	51–75%
	4	76–100%

IMMUNOHISTOCHEMISTRY

Serial sections were deparaffinized and rehydrated for routine immunohistochemistry (IHC) to detect expression of CD3 (1:20 dilution; P. Moore, UC Davis, Davis, CA) in T cells and CD79a (1:125 dilution; Clone HM57, Dako, Carpinteria, CA) in B cells. Serial sections from paraffin tissue blocks from 14 dogs were cut for immunophenotyping analysis (Fig 1-2).

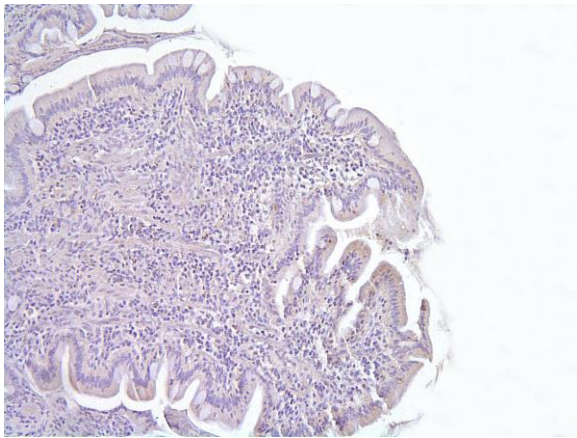


Figure 1

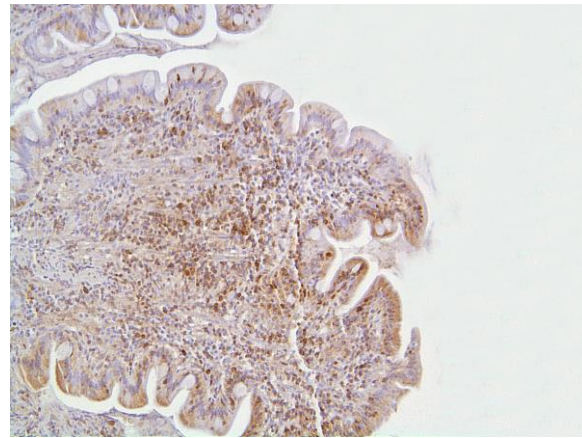


Figure 2

Fig 1-2 Immunohistochemical staining of biopsy specimen taken from dog N°17. (1) Few neoplastic lymphocytes are stained by B cell marker, CD79a. (2) Many neoplastic lymphocytes are positively stained by T cell marker, CD3.

10. STATISTICAL ANALYSIS

Diagnostic performances of the different techniques and the different observers were calculated. Since, to our knowledge, no universally accepted gold standard has been defined to differentiate IBD from intestinal lymphoma using endoscopic biopsies we decided to blindly compare all the cytological and histological results with the final diagnosis drawn by the specialist in gastroenterology according to all the results of clinical examination, endoscopy, extemporaneous cytology, histopathology and confirmed with follow-up and response to treatment. All the techniques were separately compared with this clinical diagnosis in order to define their performance and reproducibility and to potentially identify some features which could be used discriminate IBD from lymphoma.

Cohen's kappa coefficient is a statistical measure of inter-rater agreement or inter-annotator agreement for qualitative (categorical) items. It is generally thought to be a more robust measure than simple percent agreement calculation since κ takes into account the agreement occurring by chance. It measures the agreement between two raters who each classify N items into C mutually exclusive categories. The first mention of a kappa-like statistic is attributed to Galton (1892), see Smeeton (1985).

The equation for κ is:

$$\kappa = \frac{\text{Pr}(a) - \text{Pr}(e)}{1 - \text{Pr}(e)},$$

where $\Pr(a)$ is the relative observed agreement among raters, and $\Pr(e)$ is the hypothetical probability of chance agreement, using the observed data to calculate the probabilities of each observer randomly saying each category. If the raters are in complete agreement then $\kappa = 1$. If there is no agreement among the raters other than what would be expected by chance (as defined by $\Pr(e)$), $\kappa = 0$.

Cohen's Kappa Index

		Test A		
		Yes	No	
Test B	Yes			0
	No			0
		0	0	0

Accumulative concordance
Cohen's Kappa

interpretation:

values < 0 as indicating no agreement and
 0–0.20 as slight
 0.21–0.40 as fair
 0.41–0.60 as moderate
 0.61–0.80 as substantial
 0.81–1 as almost perfect agreement.

The positive predictive value, or precision rate is the proportion of positive test results that are true positives. It is a critical measure for the performance of a diagnostic method, as it reflects the probability that a positive test reflects the underlying condition being tested for. Its value does however depend on the prevalence of the outcome of interest, which may be unknown for a particular target population.

The Positive Predictive Value is defined as:

$$PPV = \frac{\text{number of True Positives}}{\text{number of True Positives} + \text{number of False Positives}} = \frac{\text{number of True Positives}}{\text{number of positive calls}}$$

The negative predictive value (NPV) is a summary statistic used to describe the performance of a diagnostic testing procedure. It is defined as the proportion of subjects with a negative test result who are correctly diagnosed. A high NPV for a given test means that when the test yields a negative result, it is most likely correct in its assessment. In the familiar context of medical testing, a high NPV means that the test only rarely misclassifies a sick subject as being healthy. Note that this says nothing about the tendency of the test to mistakenly classify a healthy subject as being sick.

The Negative Predictive Value is defined as:

$$NPV = \frac{\text{number of True Negatives}}{\text{number of True Negatives} + \text{number of False Negatives}} = \frac{\text{number of True Negatives}}{\text{number of Negative calls}}$$

The following diagram illustrates the relation positive predictive value, negative predictive value, sensitivity, and specificity.

Condition (as determined by " <u>Gold standard</u> ")				
Condition Positive	Condition Negative			
Test Outcome	Test Outcome Positive	True Positive	False Positive (<u>Type I error</u>)	Positive predictive value = $\frac{\Sigma \text{ True Positive}}{\Sigma \text{ Test Outcome Positive}}$
	Test Outcome Negative	False Negative (<u>Type II error</u>)	True Negative	Negative predictive value = $\frac{\Sigma \text{ True Negative}}{\Sigma \text{ Test Outcome Negative}}$
		<u>Sensitivity</u> = $\frac{\Sigma \text{ True Positive}}{\Sigma \text{ Condition Positive}}$	<u>Specificity</u> = $\frac{\Sigma \text{ True Negative}}{\Sigma \text{ Condition Negative}}$	

11. RESULTS

Of the 20 dogs with intestinal lymphoma, the most common breeds were Boxers (3), Beagles (3) and mongrels (2). Mean age in the lymphoma group was 8,2 years. The clinical presentation of dogs with intestinal lymphoma in this study was similar to those of dogs in previous reports, ranging from nonspecific symptoms such as anorexia and lethargy to more specific gastrointestinal symptoms such as vomiting and hemorrhagic diarrhea. In particular, the frequencies of clinical findings attributed to the intestinal tumor were: vomiting 61%, weight loss 100% (fig3) and diarrhea 100% (fig 4), of which 72% was considered severe. Recent history revealed a progressive worsening of clinical signs, not responding to symptomatic therapy or relapsing rapidly after an initial improvement. The mean time between the onset of clinical signs and the clinical presentation was 5,26 weeks, which is much shorter than the same time interval of dogs with IBD: in our study, this time interval was longer than 3 months. The CBC of dogs with intestinal lymphoma showed mild to marked anemia in 88% of cases, leukocytosis with left shift in 25% of cases. Hypoalbuminemia was a common abnormal biochemical finding: 72% of the dogs with intestinal lymphoma had a low serum albumin concentration, with most of them (84%) having less than 2 g/dL (reference interval, 3.1–4.1 g/dL).

All dogs with intestinal lymphoma were normocalcemic, when adjusted for concurrent hypoalbuminemia; 30% of the dogs had hypocoesterolemia and 70% had hypocobalaminemia.

Abdominal radiography showed non-specific findings whereas abdominal ultrasonography showed an enteritis-like aspect (70%) with an increased thickness of the intestinal wall and mesenteric lymphadenopathy (45%) (fig5). In 20% of patients, the ultrasonographic exam was normal.

The endoscopic examination showed a clobbed paving aspect of the mucosa in 50% of the patients (fig6).

Fig.3



Fig4

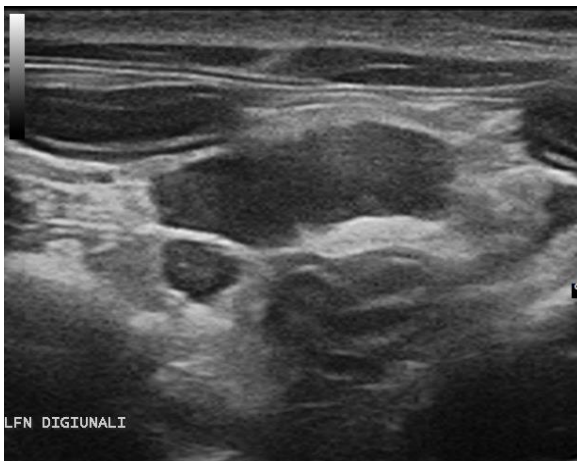
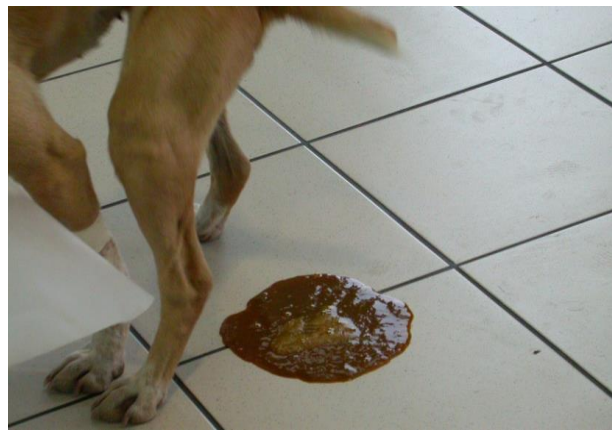


Fig5



Fig6

11.1 Cytological Results

The present study included 71 dogs that suffered from gastrointestinal symptoms: 20 cases of enteropathy-type lymphoma and 52 cases of inflammatory enteropathy.

The cytological samples were independently reviewed and subjected to a blind study from two clinical pathologists with different experience, although not expert in gastroenterology. The casistic included 20 cases of enteropathy-type lymphoma and 52 cases of inflammatory enteropathy. Cytologic smears were evaluated, drawing a tentative diagnosis according to the cytomorphological aspect.

Table 1: Review from 2 pathologist of 20 cases of enteropathy-type lymphoma

PATIENT	A	B	FINAL DIAGNOSIS
Patient 1	ENTERITIS	ENTERITIS	LYMPHOMA
Patient 2	ENTERITIS	LYMPHOMA	LYMPHOMA
Patient 3	LYMPHOMA	ENTERITIS	LYMPHOMA
Patient 4	ENTERITIS	LYMPHOMA	LYMPHOMA
Patient 5	ENTERITIS	LYMPHOMA	LYMPHOMA
Patient 6	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 7	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 8	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 9	ENTERITIS	ENTERITIS	LYMPHOMA
Patient 10	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 11	ENTERITIS	ENTERITIS	LYMPHOMA
Patient 12	ENTERITIS	LYMPHOMA	LYMPHOMA
Patient 13	ENTERITIS	LYMPHOMA	LYMPHOMA
Patient 14	ENTERITIS	ENTERITIS	LYMPOMA
Patient 15	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 16	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 17	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 18	ENTERITIS	ENTERITIS	LYMPHOMA
Patient 19	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 20	LYMPHOMA	ENTERITIS	LYMPHOMA

During the first review the observer A and the observer B showed a sensitivity of 50 percent and 65 percent respectively. For both of them the specificity was 100 percent (box1). The statistics included 20 cases of enteropathy-type lymphoma and 52 cases of inflammatory enteropathy This means that no dogs with enteritis were identified as having lymphoma.

(box1)

$$\text{Sens.} = \frac{VP}{VP+FN}$$

$$\text{Spec.} = \frac{VN}{FP+VN}$$

A (expert):

$$\text{Se} = \frac{10}{10+10} = 50\%$$

$$\text{Sp} = \frac{52}{0+52} = 100\%$$

B : $\text{Se} = \frac{13}{13+7} = 65\%$

$$\text{Sp} = \frac{52}{0+52} = 100\%$$

		Test A		
		+	-	
Test B	+	10	3	13
	-	0	59	59
		10	62	72
K Cohen				0,845

→ EXCELLENT
CONCORDANCE

All the cytomorphological features identified were reviewed in order to define the most relevant in lymphoma samples. According to the results of this first phase three main features were identified as mainly associated to lymphoma. Thus, in the second part of the study we have blindly re-evaluated the same samples using an interpretative algorithm based on the following selected cytomorphological criteria: 1) presence of lymphoid blast cells, 2) membrane alterations of the lymphoblasts, 3) presence of lymphoglandular bodies (results are expressed in Table 2)

Table 2: 2° review from 2 pathologist of 20 cases of enteropathy-type lymphoma

PATIENT	A	B	FINAL DIAGNOSIS
Patient 1	ENTERITIS	ENTERITIS	LYMPHOMA
Patient 2	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 3	LYMPHOMA	ENTERITIS	LYMPHOMA
Patient 4	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 5	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 6	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 7	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 8	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 9	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 10	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 11	ENTERITIS	ENTERITIS	LYMPHOMA
Patient 12	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 13	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 14	ENTERITIS	LYMPHOMA	LYMPOMA
Patient 15	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 16	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 17	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 18	ENTERITIS	ENTERITIS	LYMPHOMA
Patient 19	LYMPHOMA	LYMPHOMA	LYMPHOMA
Patient 20	LYMPHOMA	ENTERITIS	LYMPHOMA

During the second review the observer A showed a sensitivity of 85 percent, while the observer B of 80 percent. For both the specificity was 100% (box2). The statistics included 20 cases of enteropathy-type lymphoma and 52 cases of inflammatory enteropathy. This means that no dogs with enteritis were identified as having lymphoma.

(box2)

Sens.= VP/VP+FN

Spec.= VN/FP+VN

A:

$$Se = 17/17+3 = 85\%$$

$$Sp = 52/0+52 = 100\%$$

B: Se= 16/16+4= 80%

$$Sp = 52/0+52 = 100\%$$

		Test A		
		+	-	
Test B	+	16	0	16
	-	1	55	56
		17	55	72
K Cohen				0,986

→ EXCELLENT
CONCORDANCE

If we consider each cytologic feature independently the results in patients with lymphoma were as follows:

For the observer A:

immature lymphoid cells analysis

$$\text{Sens.} = \text{VP} / \text{VP} + \text{FN}$$

$$\text{Spec.} = \text{VN} / \text{FP} + \text{VN}$$

$$\text{Se} = 18 / 18 + 2 = 90\%$$

$$\text{Sp} = 50 / 2 + 50 = 96\%$$

membrane alterations of the lymphoid blasts

$$\text{Sens.} = \text{VP} / \text{VP} + \text{FN}$$

$$\text{Spec.} = \text{VN} / \text{FP} + \text{VN}$$

$$\text{Se} = 13 / 13 + 7 = 65\%$$

$$\text{Sp} = 45 / 7 + 45 = 87\%$$

lympho-glandular bodies

$$\text{Sens.} = \text{VP} / \text{VP} + \text{FN}$$

$$\text{Spec.} = \text{VN} / \text{FP} + \text{VN}$$

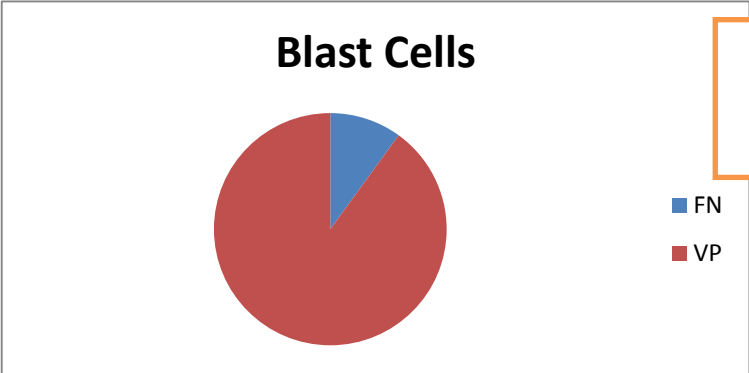
$$\text{Se} = 12 / 12 + 8 = 60\%$$

$$\text{Sp} = 44 / 8 + 44 = 85\%$$

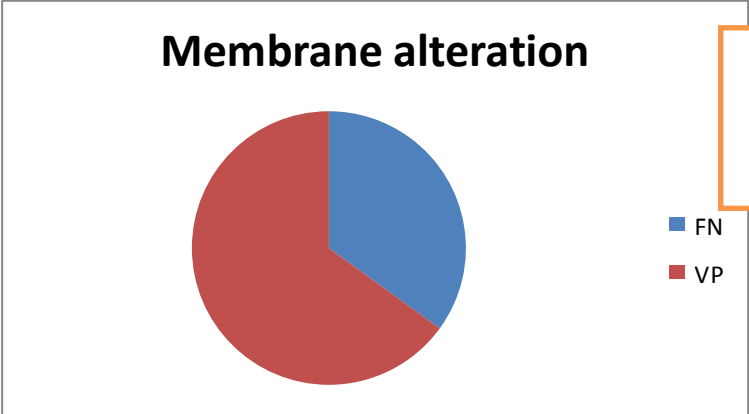
Table 3: scoring of observer A in 20 cases of lymphoma, using the 3 main cytological diagnostic features. observer A

PATIENT	diagnosis	blast	membrane	Ig bodies
Patient 1	ENTERITIS	NO	NO	NO
Patient 2	LYMPHOMA	YES	NO	NO
Patient 3	LYMPHOMA	YES	YES	NO
Patient 4	LYMPHOMA	YES	NO	YES
Patient 5	LYMPHOMA	YES	YES	YES
Patient 6	LYMPHOMA	YES	YES	YES
Patient 7	LYMPHOMA	YES	YES	NO
Patient 8	LYMPHOMA	YES	YES	YES
Patient 9	LYMPHOMA	YES	NO	YES
Patient 10	LYMPHOMA	YES	YES	NO
Patient 11	ENTERITIS	NO	NO	NO
Patient 12	LYMPHOMA	YES	YES	YES
Patient 13	LYMPHOMA	YES	YES	NO
Patient 14	ENTERITIS	YES	YES	YES
Patient 15	LYMPHOMA	YES	YES	NO
Patient 16	LYMPHOMA	YES	YES	YES
Patient 17	LYMPHOMA	YES	YES	YES
Patient 18	ENTERITIS	YES	NO	YES
Patient 19	LYMPHOMA	YES	YES	YES
Patient 20	LYMPHOMA	YES	NO	YES

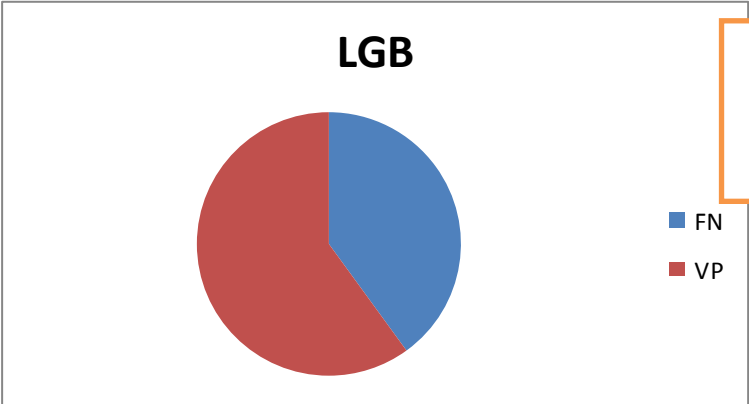
For the observer A, the positive and negative predictive values of each diagnostic criterion both in IBD and lymphoma patients were as follows:



VPP= 82%
VPN=96%



VPP= 87%
VPN=85%



VPP= 92%
VPN=86%

For observer B (expert) the results, in patients with IBD and lymphoma, were as follows:

immature lymphoid cells analysis

$$\text{Sens.} = \text{VP} / \text{VP} + \text{FN}$$

$$\text{Spec.} = \text{VN} / \text{FP} + \text{VN}$$

$$\text{Se} = 16 / 16 + 4 = 80\%$$

$$\text{Sp} = 50 / 4 + 50 = 93\%$$

membrane alterations of the lymphoid blasts

$$\text{Sens.} = \text{VP} / \text{VP} + \text{FN}$$

$$\text{Spec.} = \text{VN} / \text{FP} + \text{VN}$$

$$\text{Se} = 15 / 15 + 5 = 75\%$$

$$\text{Sp} = 47 / 5 + 47 = 90\%$$

lympho-glandular bodies

$$\text{Sens.} = \text{VP} / \text{VP} + \text{FN}$$

$$\text{Spec.} = \text{VN} / \text{FP} + \text{VN}$$

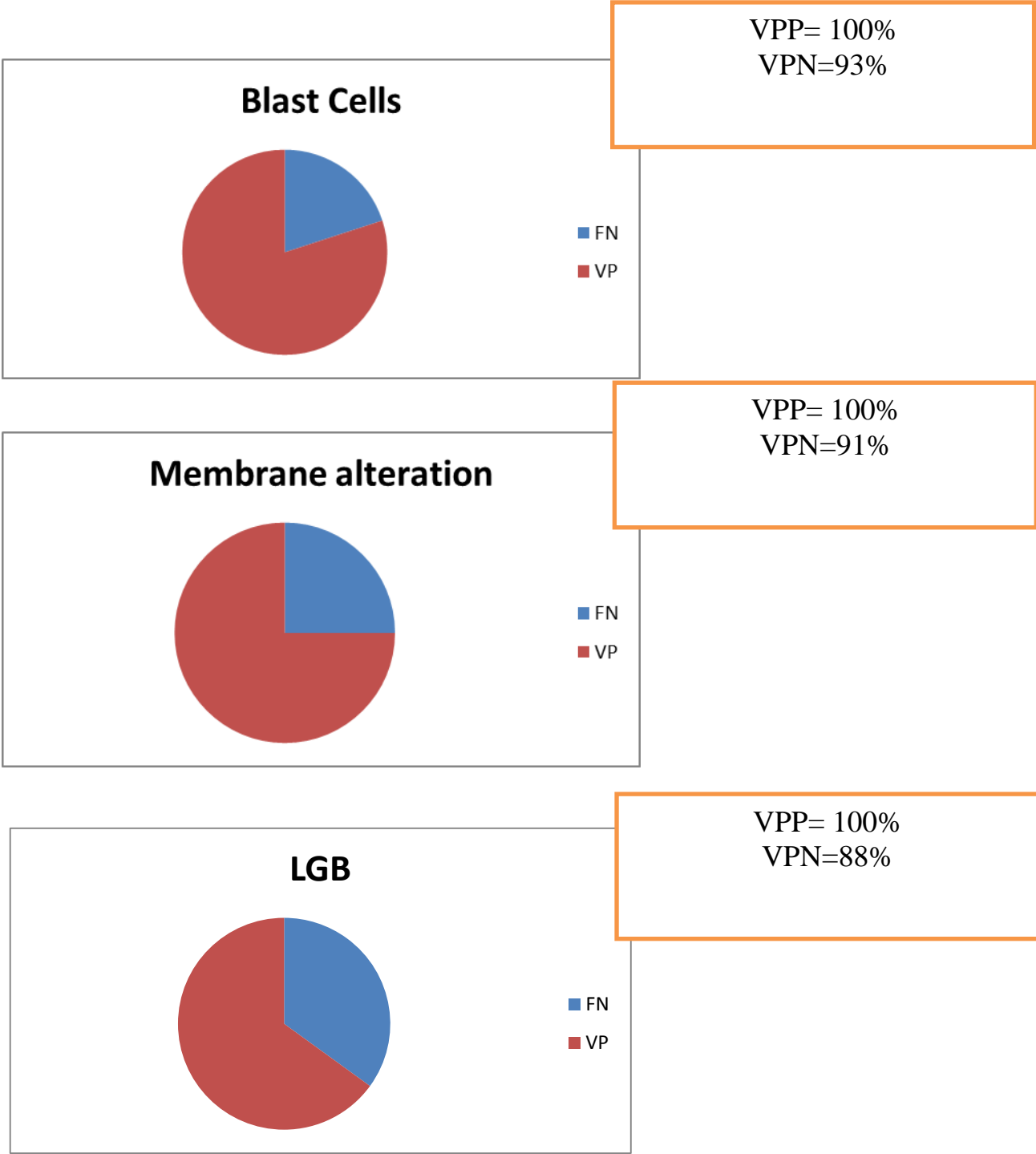
$$\text{Se} = 13 / 13 + 7 = 65\%$$

$$\text{Sp} = 45 / 7 + 45 = 87\%$$

Table 4: scoring of observer A in 20 cases of lymphoma, using the 3 main cytological diagnostic features. observer B

PATIENT	diagnosis	blast	membrane	Clg
Patient 1	ENTERITIS	NO	NO	NO
Patient 2	LYMPHOMA	YES	YES	NO
Patient 3	ENTERITIS	YES	YES	NO
Patient 4	LYMPHOMA	YES	NO	YES
Patient 5	LYMPHOMA	YES	YES	YES
Patient 6	LYMPHOMA	NO	YES	YES
Patient 7	LYMPHOMA	YES	YES	NO
Patient 8	LYMPHOMA	YES	YES	YES
Patient 9	LYMPHOMA	YES	NO	YES
Patient 10	LYMPHOMA	YES	YES	NO
Patient 11	ENTERITIS	NO	NO	NO
Patient 12	LYMPHOMA	YES	YES	YES
Patient 13	LYMPHOMA	YES	YES	NO
Patient 14	LYMPHOMA	YES	YES	YES
Patient 15	LYMPHOMA	YES	YES	YES
Patient 16	LYMPHOMA	YES	YES	YES
Patient 17	LYMPHOMA	YES	YES	YES
Patient 18	ENTERITIS	NO	YES	YES
Patient 19	LYMPHOMA	YES	YES	YES
Patient 20	ENTERITIS	NO	NO	YES

For observer B, the positive and negative predictive values of each diagnostic criterion both in IBD and lymphoma patients were as follows:



The next tables show the disaggregate concordance index of three morphological criteria between observers A and B:

Blast cells: Cohen's Kappa

		Test A		
		+	-	
Test B	+	16	0	16
	-	2	2	4
		18	2	20

Cohen's K=0.615 →GOOD

Membrane alteration : Cohen's Kappa

		Test A		
		+	-	
Test B	+	13	2	15
	-	0	5	5
		13	7	20

Cohen's K=0.765 →GOOD

LGB: Cohen's Kappa

		Test A		
		+	-	
Test B	+	11	2	13
	-	1	6	7
		12	8	20

Cohen's K=0.681 → GOOD (0,61-0,80)

11.2 Histological results

Fourteen histological samples of dogs with lymphoma were available for reviewing by a reference pathologist (table 5). HE-stained sections were blindly analyzed by a pathologist in parallel with immunohistochemistry preparations for CD3e and CD79a. I evaluate the diagnostic accuracy of gold standard and histologic results for the diagnosis of lymphoma (79%).

Table 5: Patient with lymphoma available for histological review

PATIENT	Gold Standard	Histological Diagnosis
Patient1	LYMPHOMA	LYMPHOMA
Patient2	LYMPHOMA	LYMPHOMA
Patient3	LYMPHOMA	LYMPHOMA
Patient5	LYMPHOMA	LYMPHOMA
Patient6	LYMPHOMA	ENTERITIS
Patient7	LYMPHOMA	LYMPHOMA
Patient8	LYMPHOMA	LYMPHOMA
Patient11	LYMPHOMA	LYMPHOMA
Patient12	LYMPHOMA	LYMPHOMA
Patient13	LYMPHOMA	LYMPHOMA
Patient14	LYMPHOMA	LYMPHOMA
Patient16	LYMPHOMA	LYMPHOMA
Patient17	LYMPHOMA	ENTERITIS
Patient18	LYMPHOMA	ENTERITIS

Accuracy = $\frac{VP + VN}{VP + VN + FN + FP} = 79\%$

Based on histologic features and immunohistochemistry for CD3 and CD79a, a diagnosis of IBD, T-cell lymphoma, or B-cell lymphoma was made using published criteria (table 6) and revealed that the majority of neoplastic lymphocytes were positive for CD3.

In our study, in 6 out of 8 dogs with alimentary lymphoma an immunophenotypic analysis confirmed a T-cell origin of the disease.

Table 6

PATIENT	Histological Diagnosis	HIC
Patient1	LYMPHOMA	LYMPHOMA(T)
Patient2	LYMPHOMA	LYMPHOMA(T)
Patient3	LYMPHOMA	ENTERITIS
Patient5	LYMPHOMA	LYMPHOMA(T)
Patient6	ENTERITIS	ENTERITIS
Patient7	LYMPHOMA	LYMPHOMA(B)
Patient8	LYMPHOMA	LYMPHOMA(B)
Patient11	LYMPHOMA	LYMPHOMA(T)
Patient12	LYMPHOMA	LYMPHOMA(T)
Patient13	LYMPHOMA	ENTERITIS
Patient14	LYMPHOMA	LYMPHOMA(T)
Patient17	ENTERITIS	ENTERITIS
Patient18	ENTERITIS	ENTERITIS

The agreement index calculated with Cohen's kappa coefficient, between histology and hic, was 0.649 (i.e., substantial).

		Histology		
		+	-	
HIC	+	8	0	8
	-	2	3	5
		10	3	13
Kappa di Cohen				0,649

12. DISCUSSION

Among the intestinal tumours of hematopoietic cell origin, lymphoma is the most common in dogs (Guillford WG and Strombeck DR, 1996) with primary intestinal lymphoma being less common than the multicentric form (Head KW et al, 2002).

Alimentary lymphoma, common in cats, usually accounts for 7% of all canine lymphomas and 5-7% of all gastrointestinal neoplasms (Theilen, G. H. and Madewell, B. R. 1979). A recent report has indicated that the majority of canine intestinal lymphoma is of T-cell origin. It has been divided in two types based on morphological characteristics of the tumour cells: small to moderate-sized lymphoma showing epitheliotropic behaviour, and moderate to large sized lymphoma with or without epitheliotropism (Coyle KA et al, 1996; Steimber H et al, 1995).

The former type has been classified as intestinal T-cell lymphoma according to the World Health Organization (WHO) classification system (Valli VE et al, 2002). MALT lymphoma, a small, usually of B-cell origin tumor, leads to the formation of submucosal mass, whereas the epitheliotropic T-cell lymphoma (also known as intestinal T-cell lymphoma) is usually multifocal and/or diffuse throughout the submucosa and the lamina propria of the intestine, especially in the small intestine, with frequent superficial ulceration and occasional transmural infiltration of the serosa (Willard, M. D et al, 2002). Lymphocytic-plasmacytic inflammation adjacent to or distant from the primary tumour can occur, and distinguishing between alimentary lymphoma and enteritis, especially lymphocytic-plasmacytic enteritis (LPE), can be difficult (Couto et al, 1989; Valli D.M. and Young, K.M. 2007). Generally, the prognosis of the alimentary lymphoma is poor as

compared to that of the multicentric form, regardless of treatment (Rassnick, K. M. et al, 2009).

Currently, no clear consensus exists regarding sex predilection for canine lymphoma. In some studies, the percentage of males affected with gastrointestinal lymphoma was higher than that of females, while no difference was found in other studies (Keller ET et al, 1993; Kiupel M et al, 1999). In our study, no significant sex difference was identified although this finding should be considered with caution because of the low number of cases examined.

In light of the clinical and symptomatic overlapping between IBD and enteropathy-type lymphoma, patients are often submitted to endoscopic examination for a definitive diagnosis. During the first stage of the disease, the enteropathy-type lymphoma is principally localized in the mucosa and the neoplastic infiltration is enough to cause an abnormal digestive function with non-specific gastro-intestinal signs. The endoscopic examination of the small bowel in the lymphoma group showed an aspect referred by gastroenterologists as cobblestone appearance of the intestinal mucosa (50 %): the intestinal mucosa looked friable, edematous, irregularly thickened and with a cobblestone appearance. In dogs with this endoscopic aspect, the cytological examination alone has shown in our study a good diagnostic accuracy in the differential diagnosis between lymphoma and IBD.

In our study, the cytological samples were independently and blindly reviewed by two clinical pathologists with different level of experience, although both of them had no specific expertise in gastroenteric cytology. During the first review, the experienced pathologist showed a lower sensitivity than the inexperienced one, whereas the specific-

ity was 100 percent for both. These results might be explained by the tendency of more experienced pathologists to be more conservative in their judgement in order to avoid false positive results. The agreement index calculated using the Cohen's kappa coefficient was 0.5 and it can be defined as a "mild" concordance.

In the second part of the cytology study we have blindly re-evaluated the same samples using an interpretative algorithm based on a reduced number of some selected cytomorphological clues defined by the expert gastroenterologist. These criteria were: 1) presence of lymphoid blast cells; 2) membrane alterations of the lymphoid blasts; 3) presence of lympho-glandular bodies. During the second review the observer A (experienced cytologist) showed a higher sensitivity compared with the observer B (inexperienced cytologist). For both cytologists the specificity was again 100 percent. The agreement index calculated with Cohen's kappa coefficient was 0.986 (i.e., "excellent" correlation).

The most sensitive and specific single cytomorphological criterion in differentiating enteric lymphoma from IBD was the presence of immature lymphoid cells (A: Se= 90%; Sp= 96% B: Se= 80%; Sp= 93%), although combining all criteria provides the best accuracy. The concordance index calculated with Cohen's kappa coefficient was good for all three criteria. In this study, the group of dogs with intestinal lymphoma had a very short median survival time (25 days) and the response to various therapeutic regimens was poor. A possible explanation for the poor response to treatment could be related to the immunophenotype of the lymphomas included in our study. Most cases of canine gastrointestinal lymphoma have been assumed to be of B-cell origin, likely based on the fact that almost all human, non-Hodgkin's lymphomas are of B-cell origin (Aster J et al,

1999; Ozaki K. et al, 2006). However, it is now known that canine gastrointestinal lymphoma is more commonly of T-cell origin (Miura T *et al*, 2004; Carter RF *et al*, 1986).

In our study, when immunophenotyping was performed, T-cell lymphoma was diagnosed in 71 percent of cases and B-cell lymphoma in 29 percent of cases. Because T-cell lymphomas may have a more aggressive biological behaviour and are less responsive to chemotherapeutic treatment, dogs with a gastrointestinal lymphoma may be less responsive to chemotherapy (Cartel *et al*, 1986). In the present study, however lymphoma subtype seemed to have no influence on survival time, although this conclusion must be interpreted with caution due to the low number of cases examined.

In conclusion, cytological examination of squash-prep samples could be very useful in addressing infiltrating enteropathy, using the proposed diagnostic criteria. The most outstanding result of our study, was the higher diagnostic accuracy of squash-prep cytology compared with histology.

Histopathology and immunohistochemistry are generally considered useful in the differential diagnosis between IBD and lymphoma.

Seventy-five percent of the alimentary lymphomas in this study stained positively for the CD3 marker, indicating a T-cell origin for these tumors in dogs, whereas only 25 percent stained positively for the B-cell marker. This is opposite to the pattern seen in humans, wherein a B-cell is the origin of nearly all non-Hodgkin's lymphomas. Several studies on humans have found the incidence of B-cell lymphoma to be from 50 up to 100 percent when compared with T-cell lymphomas (Hansen PB *et al* 1998; Parodi AL *et al*, 1988; Teske E *et al*, 1994; Vinzio S *et al*, 1998). Primary gastrointestinal T-cell lymphomas are so rare in humans that several studies either do not address T versus B-cell as a prog-

nostic indicator, even though tumors were identified by the immunotype (Hansen PB et al 1998; Parodi AL et al, 1988), or all the tumors in the study were of B-cell origin (Whooley BP et al, 1998).

In our findings however, although the evaluation of histopathologic results is not within the aims of this study, the diagnostic performances of histopathology were not better than those of cytology alone. In our view, this stresses the possible role of cytology for diagnosing intestinal lymphoma and suggests that a wider panel of tests including cytology, histology, immunophenotyping and possibly molecular biology techniques (PCR for antigen receptor rearrangement or PARR) is mandatory to address the problems of a differential diagnosis.

In our view, only an accurate evaluation of both clinical, imaging and laboratory results by a specialist in gastroenterology may bring to a final diagnosis and appropriate treatment and prognosis.

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